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This award was used to train Dr. Minhong Yan during his Ph.D. research, and as such has been highly successful. Dr. Yan received his Ph.D. in May of 1997, and began a Post-Doctoral fellowship in July 1 1997. In experiments performed under this award, Dr. Yan first mapped sites of phosphorylation on the MEK1 protein kinase, and next identified the role of the MEKK1 protein kinase in signal transduction, placing it upstream of the SEK1 kinase and the Stress-Activated Protein Kinase. MEKK1 directly phosphorylates tSEK1, which in turn phosphorylates SAPK. Subsequent to accomplishment of this Aim, Dr Yan has identified a novel activity of MEKK1, specifically a tyrosine kinase actvitity associated with the MEKK1 kinase (thought to be exclusively a serine/threonine kinase heretofore) as well as a cellular substrate of MEKK that becomes phosphorylated on tyrosine to undetermined ends.

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INTRODUCTION

The precise mechanism by which cell growth-stimulatory signals are propagated remains a central and largely unanswered question. Much progress has been made in characterizing the structure and function of membrane-bound receptors of growth factors, as well as identification of many of these receptors as tyrosine kinases. In 1983, Cooper and Hunter identified the Microtubule Associated Protein II kinase (MAP kinase) as a major phosphotyrosine containing protein that became phosphorylated in response to mitogen treatment or cell transformation, and suggested that MAP kinase was possibly a direct substrate of a receptor tyrosine kinase or the *src* oncoprotein kinase. This protein was later rechristened Mitogen Activated Protein kinase, yielding the same acronym. An alternate nomenclature designates MAP kinase as the Extracellular signal Regulated Kinase, or ERK.

Surprisingly, MAP kinase proved not to be a direct substrate of receptor tyrosine kinases, but rather was a substrate of a serine/threonine and tyrosine kinase that itself was several steps downstream of the activated receptor kinase. In recent years, some molecular details have been worked out, but a large knowledge gap remains, including the means by which the signal from receptor tyrosine kinases passed to freely soluble kinases within the cytoplasm, and subsequently is passed onto nuclear growth control proteins.

MAP kinase is activated by phosphorylation in response to numerous mitogenic events, (as reviewed recently). Two activators of MAP kinase have been characterized, one termed MEK (MAP kinase, or Erk Kinase; alternatively MAP kinase-kinase-1) and the other MAP kinase-kinase-2. MEK is in turn activated by phosphorylation by the v-*raf* oncoprotein. Activation of the c-*raf* oncoprotein is stimulated by the *ras* activation pathway, and recently direct interaction of c-*raf* with the *ras* oncoprotein has been demonstrated.

Additionally, a separate signal impinges upon the MAP kinase activation pathway, arising not from growth factor receptor tyrosine kinases, but from an interaction with heterotrimeric G proteins associated with 7-membrane spanning receptors. This central regulating kinase, termed MEK kinase (or MEKK) because it lies immediately upstream of MEK, has only recently been cloned, although several groups have characterized the enzymatic activity (see for review). While the input signal that passes through MEKK originates separately from the signal that passes through *raf*, the net effect, i.e. activation of MAP kinase, seems equivalent. Another observation that these two signals may be equivalent is that like oncogenic activation of the *ras* oncoprotein, a heterotrimeric G protein (gip2) can be oncogenically activated by mutation. While the downstream mediators of gip2 have not been identified, at this point it seems likely that they involve activation of MEKK or related regulatory kinases, since cell transformation by gip2 results in activation of MAP kinase.

Once MAP kinase becomes activated, it presumably phosphorylates additional proteins significant for transmission of the mitogenic stimulus. Some of these substrates may have been identified; for example, c-PLA2 is a known substrate of MAP kinase and phosphorylation of this substrate may be relevant to further propagation of signals via phospholipid release leading to PKC activation. Our interest was piqued by recent reports that demonstrate that activated MAP kinase becomes translocated to the nucleus after stimulation. This fact raises the obvious possibility that MAP kinase may have direct nuclear substrates relevant to growth stimulation. Some of these substrates have potentially been identified, for example lamin and the *jun* transcription factor have been reported to be phosphorylated by MAP kinase. As shown below, our group has recently demonstrated that the tumor suppressor protein pRb, is also phosphorylated and

functionally regulated by MAP kinase. Thus, MAP kinase may terminate the cytoplasmic component of the signal transduction cascade, resulting in nuclear phosphorylation that regulate key growth stimulatory processes.

Several unanswered questions arise from these observations.

- What are the physiologically significant substrates of this cascade?
- Do different mitogenic signals involve distinct, though possibly related, kinases? How are these input signals related? Does this diversity result in a specificity of response of particular tissues?
- How are components of this kinase cascade controlled via phosphorylation? A precise characterization of these phenomena could clarify the molecular interactions between kinase and substrates, and could provide valuable reagents to further characterize their role in signal transduction.

BODY

Part 1- Identification of two serine residues of MEK-1 that are differentially phosphorylated during activation by *raf* and MEKK

Cell growth signal transduction is frequently accompanied by phosphorylation and activation of MAP kinase, as reviewed recently (1). Two kinases capable of activating MAP kinase have been cloned, one termed MEK-1 (MAP kinase or Erk Kinase; alternatively MAP kinase-kinase-1) (2, 3) and the other MAP kinase-kinase-2 (4). MEK-1 is in turn activated by phosphorylation by the v-*raf* oncoprotein (5). Activation of c-*raf* is stimulated by the *ras* activation pathway, and recently direct interaction of c-*raf* with the *ras* oncoprotein has been demonstrated (6, 7, 8, 9)

A kinase structurally unrelated to *raf* is similarly able to phosphorylate and activate MEK-1. This kinase, termed MEK kinase (or MEKK) because it lies immediately upstream of MEK, has recently been cloned (10), and several groups have characterized the enzymatic activity (see (11) for review). While the growth signal that passes through MEKK might originate separately from the signal that passes through *raf*, the net effect, i.e. activation of MAP kinase, seems equivalent (10) .

Since MEK-1 lies at the convergence of signaling pathways that may have distinct origins, the molecular details of MEK regulation are important. We have investigated the nature of MEK-1 activation by *raf* and MEKK, and have developed *in vivo* and *in vitro* systems to model the activation of MEK. Using these model systems, we have identified the amino acid residues upon which *raf* and MEKK phosphorylate MEK-1, and show here that these sites are necessary for activation of MEK-1 *in vitro* and *in vivo*.

Analysis

Identification of the sites of activation of MEK-1 clarifies the role of MEKK and *raf* in transduction of cellular growth signals. The two sites of activating phosphorylation lie within a domain of MEK-1 between kinase domains VII and VIII(15). This activation domain of MEK-1 is conserved between MEK variants and species homologs. Figure 8 shows the alignment of the comparable region of many MEK homologs across diverse species. In all cases, the two serines identified as MEK-1 activation sites are preserved, although in yeast and Xenopus the second serine is changed to a threonine residue.

Figure 8B depicts the analogous regions of several serine-threonine protein kinases for which the sites of activating phosphorylation are known are shown. In all examples found, activating phosphorylation also occurs between conserved kinase domains VII and VII. For the analogous activating region of MAPK, this region lies in a solvent-exposed portion of the protein, which has been termed the "activating lip"(16), and which may partially obstruct a substrate binding pocket. Spacial conservation of the sites of activating phosphorylation suggests that this mode of regulation of kinase activity is strongly conserved, especially among kinases within signal transduction cascades. Activation sites in other kinases might thus be inferred by homology to this region.

The constitutive activity of our MEK-2E allele strongly supports the identification of codons 218 and 222 as sites of activating phosphorylation on MEK. Further, this allele could prove to be a valuable reagent for analysis of signal transduction events, since is almost certainly contributes a continuous MEK-1 signal to the cell. We have recently constructed cell lines that express the MEK-2E allele in a regulated fashion, and are beginning to measure the effects of this allele on growth signaling in cells. It should be noted that our strategy of substituting acidic residues in place of activating phosphorylation sites is not always successful. As a pertinent example, we have detected no activity in alleles of MAPK (obtained from Michael Weber) containing substitutions of glutamic acid for either the phosphorylated threonine, or tyrosine residues, or both.

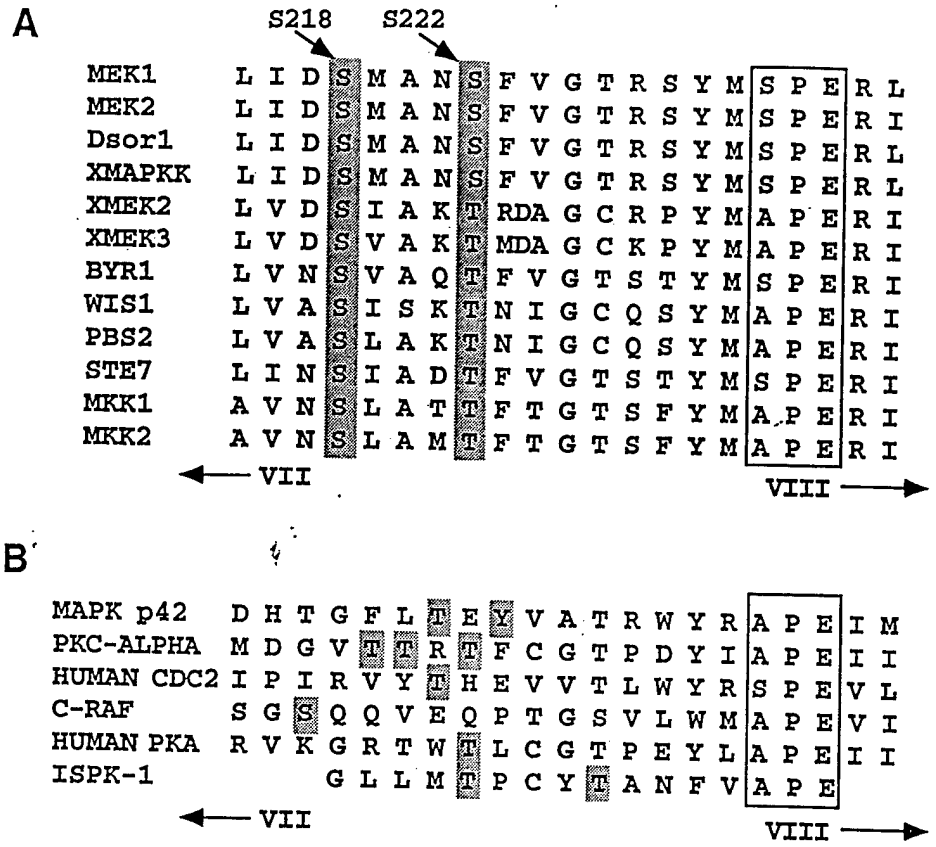

Figure 8. Comparison of the activation domain in homologs of MEK-1 and in other serine/threonine kinases.

Panel A: Amino acid sequences of MEK-1 and homologous proteins are compared in the region of the activating phosphorylation sites of MEK-1. Shaded boxes indicate serine or threonine residues conserved at the sites of MEK activation. Amino acid sequences of rat MEK-2 (MAP-kinase-kinase-2) (4) Drosophila Dsor-1 (19), Xenopus MAPKK (20) , Xenopus MEK-2 and MEK-3 (21), Schizosaccharomyces byr-1 (22) and wis-1 (23), Saccharomyces PBS-2 (24), STE7 (25), and MKK1 and MKK2 (26) were from published sources.

Panel B: Regions of several serine/threonine protein kinases between conserved domains VII and VIII are compared in which the kinases are known to be stimulated by phosphorylation in this region. Shaded boxes show sites of phosphorylation. Published sources identifying activating sites were used for MAPK(27), PKC-α(28), cdc2(29, 30),c-*raf*(31), PKA(32), and ISPK-1(33).

While MEK-1 is clearly activated by *raf* and MEKK, it is also capable of phosphorylating itself, as documented by several groups (17, 18) . Using our S218T and S222T mutants we have confirmed that MEK-1 autophosphorylates both codons 218 and 222, and also phosphorylates uncharacterized threonine and tyrosine residues.

Our studies have shown clearly that phosphorylation of MEK-1 by MEKK and *raf* are not identical, with MEKK strongly preferring codon S218 as a site of phosphorylation. However, we do not know if this difference is reflected in biological differences in the signaling process effected by these two kinases. Using phosphorylation and activation of MAPK as a measure of MEK-1 function, both MEKK and *raf* seem equally able to activate MEK-1. The differences we observe in site preference might, however, reflect biological differences in signaling in several ways. For example, the two distinctly phosphorylated forms of MEK could recognize different substrates other than MAPK. Under this scenario, doubly-phosphorylated MEK (activated by *raf*) could phosphorylate an unknown substrate *critical for cell transformation,* that is not recognized by MEKK activated MEK-1. Alternatively, one of the differently phosphorylated MEK-1 forms could remain activated longer within the cell. Since phosphatase(s) that inactivate MEK-1 have not been characterized, it is possible that a separate phosphatase is responsible for dephosphorylating each residues. If this were true, termination of the two activating events might be separately regulated. Since our data indicate that singly phosphorylated MEK-1 remains partially activated (see Figure 4) a phosphatase specific for codon 218 could specifically negate signals arising from MEKK activation, while leaving the signal arising from *raf* partially intact.

Irrespective of mechanism, the differences between MEK-1 signal transduction effected by MEKK or *raf* is significant for one overriding reason: *raf* has clearly been identified as a component of an oncogenic kinase cascade, while MEKK has not. Alternative phosphorylation of MEK-1 protein, resulting in similar yet distinct activation of MEK-1, could be a means by which oncogenic versus non-oncogenic growth signals are propagated.

Since this work was completed, a report identifying MEK codons 218 and 222 as substrates for *raf* has been published (19).

Results

Expression of signal transduction kinases

We used the T7-polymerase gene expression system of Moss (12, 13) to express MAP kinase (p42), MEK-1, and truncated, kinase active versions of MEKK and *raf* either alone or in combinations within cells. To monitor expression of these protein kinases, and to afford simple means of micropurification of the wild type or mutant forms, we modified each of the proteins by the addition of a 10 amino acid synthetic epitope to the amino terminus of each protein. Each of the kinases is thus identifiable by western blotting against the synthetic epitope (termed EE), and can be immunopurified under non-denaturing conditions.

When expressed alone using this system, MAPK is almost completely unphosphorylated (as shown in Figure 1, lane 1) and phosphorylation of MAPK is unaffected by co-expression of MEK-1 (lane 2). This is probably because MEK itself is dependent upon upstream activation. When MAPK is expressed together with either Δ*raf* or ΔMEKK, increased phosphorylation of MAPK is seen, evidenced by a band with reduced electrophoretic mobility. Inclusion of MEK with these kinases results in nearly complete phosphorylation of MAPK (lanes 4 and 6), apparently due to expression of a complete activation pathway. In these lanes, MEK also shows a reduced electrophoretic migration compared to when it is expressed alone. This change reflects phosphorylation of MEK on serine and threonine residues (14) possibly from phosphorylation by Δ*raf* or ΔMEKK, or by retrograde phosphorylation of MEK by MAPK or other kinases.

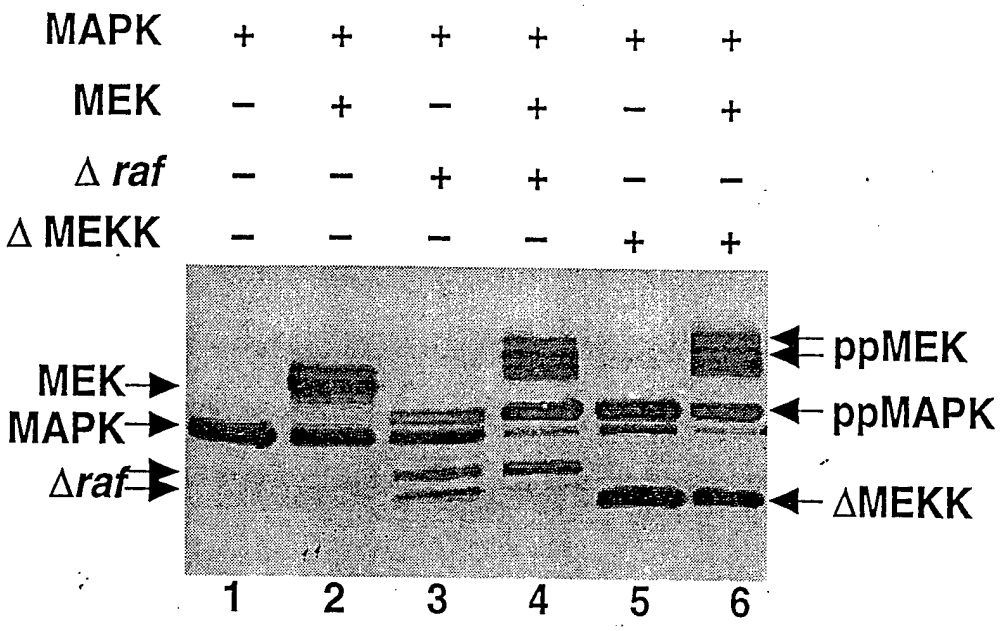

Figure 1. *in vivo* modeling of MAPK and MEK-1 activation.

MAPK was expressed in CV1 cells together with MEK-1 with or without activated forms of MEKK or *raf* as indicated. Each of the kinases were tagged with an amino terminal synthetic epitope ("EE") , and the expression of each protein was monitored using anti-EE antibody for immunoblotting. MAPK expressed alone (lane 1) is minimally phosphorylated, as evidenced by the absence of the slowly migrating phosphorylated form. Coexpression of MEK with MAPK (lane 2) is insufficient to increase MAPK phosphorylation, unless an activating kinase such as *raf* (lane 3 and 4) or MEKK (lane 5 and 6) is included. In this case, MEK displays several forms with reduced mobility consistent with phosphorylation. MAPK is phosphorylated in response to the *raf* or MEKK activators, even more so in conjunction with MEK-1. Additionally, Δ*raf* shows a slowly migrating phosphorylated band, especially when expressed together with MEK (lane 4).

in vitro phosphorylation of MEK-1 by MEKK and *raf*

Activation of MEK-1 was also modeled *in vitro* using the active forms of MEKK or *raf* to phosphorylate MEK-1. To do this, we immunopurify epitope tagged forms of both of these kinases (see Experimental Procedures) and similarly purified a kinase inactive mutant of MEK-1 (containing a lysine to arginine mutation within the ATP binding domain of the kinase (15)). An example of this is shown in Figure 2, in which both ΔMEKK and Δ*raf* are used to phosphorylate MEK-KR protein (Lanes 1 and 2), while the preparation of substrate MEK-KR is itself completely devoid of phosphorylating activity (Lane 3). ΔMEKK is also strongly phosphorylated, apparently through an autocatalytic reaction, while Δ*raf* is weakly phosphorylated (Lane 2, but more clearly seen in Figure 4). In experiments not shown, we found that MAPK phosphorylated MEK-1 on serine and threonine residues, while MEKK and *raf* phosphorylated exclusively serine residues. Since *raf* and MEKK are able to phosphorylate MEK-1 only on serine residues, and this phosphorylation was sufficient to activate MEK kinase activity (see Figure 4 and 5 below), we focused on serine residues as potential sites of activating phosphorylation.

Identifying the sites of MEK-1 phosphorylation by *raf* and MEKK

Inspection of the predicted amino acid sequence of the rat MEK-1 protein reveals 18 serine residues, of which some are not conserved in MEK alleles or the homologous counterparts of MEK in other species. If activation of MEK-1 is conserved through its evolution, we predicted that the significant serine residues should either be identical in other alleles, or possibly could be altered to threonine residues. Based on these suppositions, we initiated a program of directed mutagenesis targeting several serine codons, preparing these mutants in a background of the inactivated K96R MEK mutant to eliminate autophosphorylation by MEK.

Phosphorylation of mutant MEK proteins using ΔMEKK or Δ*raf* kinases, showed that mutant S218A demonstrated reduced phosphorylation despite equivalent expression levels (not shown). However, mutation of this residue might have caused unpredictable changes in protein conformation, resulting in decreased phosphorylation at a distant site. To confirm phosphorylation of serine 218 genetically we mutated this codon to a threonine residue. The level of phosphorylation of mutant protein S218T by ΔMEKK was equivalent to that of the wild type protein. However, about 80% of the radiophosphate in this mutant protein was localized to threonine residues with the remainder on serine residues. Because a small amount of phosphoserine was still detected in mutant S218T, we surmised that at least one more site of serine phosphorylation was present on MEK.

We identified the additional phosphorylation site based on two observations. First, one of two chymotryptic peptides obtained during peptide mapping of the *in vitro* labeled MEK S218T mutant contained both phosphoserine and phosphothreonine (data not shown). This suggested that the two phosphorylation sites were contained within the same chymotryptic peptide. Second, the S218 residue lies within a domain similar to one in MAP kinase phosphorylated on two clustered residues (see figure 8). This lead us to speculate that MEK might be doubly phosphorylated in a similar cluster. We subsequently mutated each of the two serine residues near S218, creating threonine codons, and found that only one of these mutant proteins (S222T) was phosphorylated on threonine. A double mutant in which both serine codons at 218 and 222 are altered to threonine contained no phosphoserine when phosphorylated by MEKK or *raf*. These experiments are shown in Figure 3.

These data show that activated *raf* and MEKK phosphorylate the same two residues on MEK-1. MEKK shows apparent preference for the S218 residue *in vitro*, while *raf* phosphorylates each site roughly equivalently, based on the levels of phosphorylation of the individual threonine mutants. This observation is examined more closely below in Figure 7. Additionally, the data in Figure 3 show that no sites other than the serines at 218 and 222 are phosphorylated, since the 218T/222T mutant displays no phosphoserine and the wild type protein displays no phosphorylation on threonine or tyrosine.

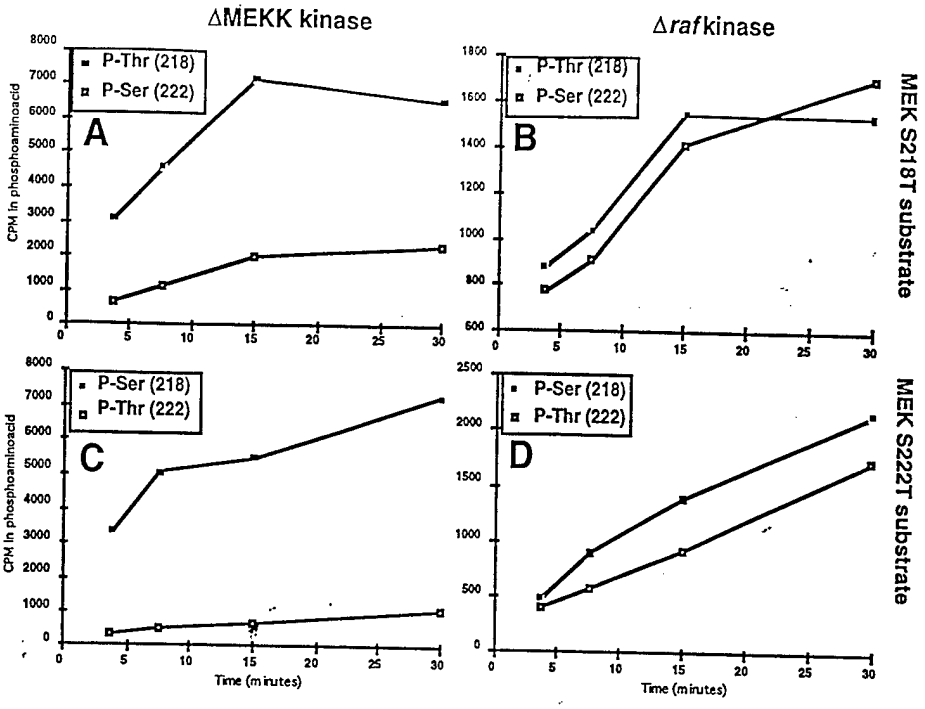

Figure 7. Time course of MEKK and *raf* phosphorylation of each activating residue.

To measure phosphorylation of MEK on codons 218 and 222, MEK S218T or S222T mutants were constructed, together with the inactivating K97R mutation of MEK-1. Each mutant MEK-1 protein was expressed separately and then immunopurified and reacted together with ΔMEKK or Δraf kinases. Parallel reactions were stopped after increasing time with SDS sample buffer, and analyzed by SDS PAGE. Radiolabelled MEK protein was subjected to phosphoamino acid analysis and the amount of radiolabel associated with phosphoserine or phosphothreonine was quantified using an AMBIS beta detector.

Four experiments are shown using either MEK S218T substrate (panels A and B) or MEK S222T (panels C and D) reacted with either ΔMEKK (panels A and C) or Δ*raf* (panels B and D) for the time shown. The amount of radiophosphoamino acid detected arising from each codon (either phosphoserine or phosphothreonine) is plotted separately. MEKK phosphorylates the residue at codon 218 more rapidly than the residue at codon 222 regardless of the hydroxy amino acid at that codon position. In contrast, Δ*raf* kinase phosphorylates both codons approximately equally at all time points.

Do the identified sites confer "activatability" to MEK-1 *in vivo?*

If S218 and S222 are required to activate MEK-1, an important prediction to test is that mutation of these sites would eliminate the ability of MEK to be activated by *raf* or MEKK *in vivo*. To test this prediction, we expressed epitope tagged MEK-1 proteins (wild type or alanine substitutions at the 218 and 222 sites, all EE tagged) with or without active Δ*raf* proteins (without the EE epitope tag in this experiment). We then specifically immunopurified the epitope tagged MEK proteins, and assayed them for MAP kinase phosphorylation activity *in vitro*. As shown in Figure 4, both of the single-site mutant proteins are still partly activated. Only the double alanine mutant demonstrated no activity either in the presence or absence of *raf*. Thus, both S218 and S222 contribute to the activatability of MEK by *raf*. The wild type MEK-1 protein was activated by co-expression with Δ*raf*, but clearly displays some basal activity even in the absence of co-transfected active *raf*. Since the plasmid-encoded proteins are expressed in the full context of normal cellular proteins, this low level of MEK activation almost certainly results from activation by uncharacterized cellular kinase(s), probably including but not exclusively the endogenous *raf* and MEKK kinases.

Do the sites confer activatability *in vitro?*

Since the activation of MEK-1 by *raf* in Figure 4 occurred in intact cells, it remained possible that activation of MEK was not a result of *raf*, but rather by an intermediate *raf*-activated kinase. To model the activation of MEK *in vitro*, we purified *raf* and MEKK and used these kinases to phosphorylate MEK or the alanine-substituted mutant, in the presence of unlabelled ATP. Phosphorylated MEK protein was then incubated with an inactive mutant (K46R) of MAPK to quantify MEK activity. As shown in Figure 5, both *raf* and MEKK is able to activate wild type MEK-1 *in vitro*, while the mutant substituting alanine residues at codons 218 and 222 is unable to be activated. Thus, these codons are required for direct activation by either *raf* or MEKK.

Mutation of the activation sites to glutamic acid results in constitutive activation

Since phosphorylation of MEK-1 at codons 218 and 222 introduces negative charges into this portion of the protein and results in kinase activation, we conjectured that substitution of negatively charged amino acids at these two positions might similarly activate a mutant MEK-1 protein. Such a constitutively active allele would be predicted to be active independent of activation by phosphorylation by upstream kinases. To test this hypothesis, we constructed a mutant allele of MEK that contains the two mutations S218E and S222E, termed MEK-2E. Immunopurified MEK-2E was found to be catalytically active (see Figure 6) but a more important question was whether the activity of the MEK-2E protein requires activation by upstream kinases.

In the experiment shown in Figure 6, we expressed MEK-WT or MEK-2E together in cells with either Δ*raf* or the kinase-inactive Δ*raf*-KR mutant (neither *raf* allele was epitope tagged in this experiment). We then measured the ability of the immunopurified MEK to phosphorylate inactive MAPK-KR, detecting phosphorylation of MAPK by upward mobility shift on SDS-PAGE. Approximately 90% of immunopurified MAPK substrate is unphosphorylated, (lane 1) when incubated without MEK protein. Incubation of the MAPK-KR substrate with Δ*raf*-activated MEK-1 results in nearly complete conversion of MAPK to the phosphorylated form (Lane 2), but little conversion of MAPK is seen if MEK-1 is co-expressed with the inactive *raf* allele, demonstrating that activity of wild type MEK-1 protein is dependent upon activation by the co-expressed *raf* kinase. The MEK-2E mutant protein is also able to effect complete conversion of the MAPK-KR substrate to the phosphorylated form, but in contrast to the wild type MEK protein, MEK-2E is active when expressed either with or without active *raf* kinase (lane 5). Thus MEK-2E activity is independent of upstream activation.

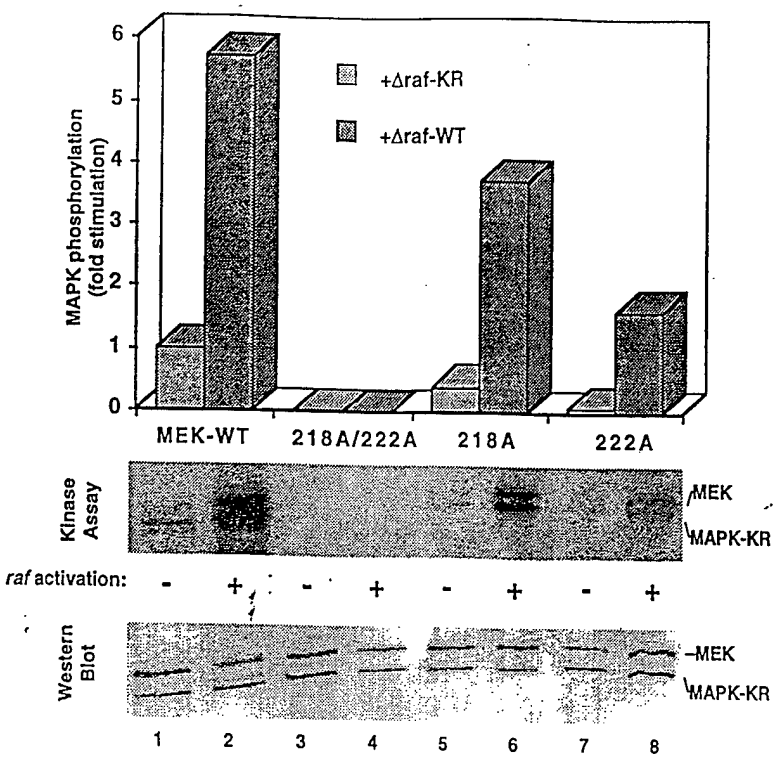

Figure 4. S218 and S222 are required for activation of MEK *in vivo.*

EE-epitope tagged MEK-WT, or alanine substitution mutants as indicated were coexpressed with Δ*raf* or the inactive mutant Δ*raf*-K375R (that both lacked the EE epitope) to assess activation of MEK by Δ*raf*. EE-tagged MEK protein was specifically immunoprecipitated using anti-EE Affigel beads, and assayed for kinase activity by incubation with inactive epitope tagged MAPK-KR substrate. Phosphorylated proteins were separated by SDS-PAGE and blotting onto Immobilon. Incorporation of radiophosphate into MAPK was quantified using an AMBIS beta-imager and is shown in the top panel. The autoradiogram of this gel is shown in the middle panel. After quantification, the filter was probed using anti-EE mAb to verify equal recovery of kinases and substrate, shown in the bottom panel. Wild type MEK was active when expressed in the presence of *raf*, but not in the presence of the inactive mutant *raf*. Mutation of both sites to alanine codons completely prevented both basal MEK activity and *raf* activation. Mutation of individual serine codons to alanine resulted in partial activation. Autophosphorylation of MEK parallels phosphorylation of the MAPK substrate.

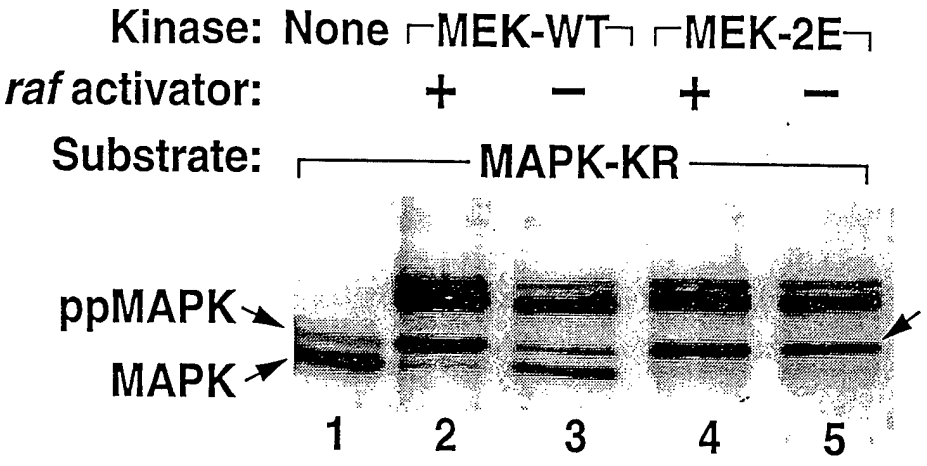

Figure 6. MEK-2E allele is constitutively active

EE-epitope tagged MEK-WT or MEK-2E proteins were expressed together in cells with untagged Δ*raf* or the kinase-inactive Δ*raf*-KR mutant (designated + or - *raf* activator). Activity of the immunopurified MEK was detected using inactive MAPK-KR as substrate, detecting phosphorylation of MAPK by upward mobility shift on SDS-PAGE . MAPK is mostly unphosphorylated when incubated without MEK protein (lane 1). Incubation of the MAPK-KR substrate with Δ*raf*-activated MEK-1 results in nearly complete conversion of MAPK to the phosphoryated form (Lane 2), but little conversion if MEK-1 is co-expressed with the inactive *raf* allele (Lane 3). The MEK-2E mutant protein is also able to effect complete conversion of the MAPK-KR substrate to the phosphorylated form, either when expressed with (Lane 4) or without (Lane 5)active *raf* kinase. Thus MEK-2E activity is independent of upstream activation.

MEKK and *raf* have distinct specificity for site phosphorylation.

The experiment shown in Figure 3 suggests that phosphorylation of MEK-1 by *raf* results in approximately equal phosphorylation on serines at codons 218 and 222, while MEKK phosphorylates codon 218 preferentially. However, this distinction could reflect differences in the completion of the phosphorylation reaction rather than differences in the actual site preferences. Therefore we sought means of observing the kinetics of phosphorylation of each codon separately.

We first considered using synthetic peptide substrates to measure the site specificity of MEKK or *raf*. However, versions of MEK-1 containing large (50 to 100 codon) deletions distant to the activating sites have been completely unphosphorylated by MEKK or *raf*. We interpret this to mean that an intact MEK protein is required for recognition of MEK-1 as a substrate by these kinases. It thus seems unlikely that peptide substrates could give meaningful results.

To measure phosphorylation of MEK on each of the two codons, we instead utilized MEK S218T and S222T mutations, together with the inactivating KR mutation. The reciprocal threonine mutations are important for measuring potential differences in *codon* specificity versus preference for serine over threonine residues. MEK S218T or 222T mutants were expressed separately and then immunopurified and reacted together with ΔMEKK or Δ*raf* kinases for increasing time periods. Radiolabeled MEK protein was subjected to phosphoaminoacid analysis and the amount of radiolabel associated with phosphoserine or phosphothreonine was quantified using an AMBIS beta detector.

Figure 7 shows the analysis of the four experiments using the two substrates and the two kinases. MEKK was found to phosphorylate the residue at codon 218 more rapidly than the residue at codon 222 in reactions with both mutant MEK proteins (Panels A and C). At later time points, phosphorylation on codon 218 plateaus and phosphorylation at codon 222 increases slightly. In contrast, Δ*raf* kinase (Panels B and D) phosphorylated both codons approximately equally at all time points. When codon 222 was substitute with threonine, slightly less radioactive phosphothreonine was detected with either kinase. This may reflect that threonine is a slightly less-preferred residue for both kinases. It is clear, however, that phosphorylation of MEK by MEKK and *raf* is biochemically distinguishable, although we have not detected biological consequences of this differentially phosphorylated MEK proteins.

Part 2– MEKK1 activates the Stress Activated Protein Kinase (SAPK) in vivo, not MAP kinase, via direct phosphorylation of the SAPK activator SEK1.

To study signaling events downstream of the MEKK protein kinase, we derived NIH3T3 cell sublines in which expression of this protein is inducible. Surprisingly, induction of MEKK in these cells results not in the activation of MAPK, but instead stimulates Stress Activated Protein Kinases (SAPKs). Purified MEKK functions in a reconstituted signaling cascade by phosphorylating and activating the newly described SAPK activator, SEK1, which itself then activates SAPK by phosphorylation. These data identify the physiological function of MEKK as an activator of a signaling cascade culminating in activation of SAPK.

In all eukaryotes a multiplicity of signal transduction cascades function to transmit complex signals to the nucleus. One of the most fully characterized of these pathways in mammalian cells involves activation of Mitogen Activated Protein Kinases (MAPKs) by a multi-step phosphorylation cascade (for review, see (20, 21, 22)). Activation of MAPK requires tyrosine and threonine phosphorylation by a MAPK kinase, termed MEK (2, 23). which itself, is also activated by phosphorylation. The first enzyme shown to capable of activating MEK was the Raf oncoprotein kinase (5, 24, 25) . Raf is activated following recruitment to the plasma membrane via its interaction with activated Ras (6, 7, 8, 9). Thus, agents that stimulate GTP loading of Ras, such as polypeptide mitogens and insulin, also stimulate MAPK activation via the intermediate actions of the Raf and MEK.

A second potential MEK kinase (termed MEKK) was cloned by virtue of its homology to a kinase involved in yeast mating signal cascades, STE11 (10). Like Raf, MEKK contains a carboxy terminal kinase domain preceded by a potential regulatory domain. Expression of the carboxy terminal kinase domain in mammalian cells results in a constitutively active protein kinase that is capable of activating MEK *in vitro* (10, 26) . High level expression of MEKK in COS cells (10) or using vaccinia virus vectors (26) is also capable of activating MEK and MAPK. By these criteria, MEKK represents an alternate means of activating the MAPK signaling pathway.

In addition to the mitogenic cascade described above, at least two families of MAPK-like kinases have recently been uncovered in mammalian cells. One of these is structurally homologous to the HOG kinase of yeast (27) that responds to osmotic shock and endotoxin (28) . Little is known about the signals that activate this kinase in mammalian cells. Another family of kinases related to MAPK was originally purified from cycloheximide treated rat liver (29) , and represents at least three related proteins of approximately 54 kDa (30) . These kinases phosphorylate the N-terminus of the Jun transcription factor specifically on serines 63 and 73 (31) , resulting in AP1 transactivation (32) . Like MAPKs, these Jun kinases require tyrosine and threonine phosphorylation for activity (33) but their activity is poorly induced by mitogens. In contrast, potent agonists include agents of cellular stress, including TNF-α treatment, UV irradiation, heat shock, ischemia, and the translational inhibitors cycloheximide and anisomycin (30, 34, 35) .The enzymes were hence termed Stress Activated Protein Kinases (SAPKs).

While MEK does not phosphorylate SAPKs, we recently identified a MEK-related protein kinase termed SAPK or ERK Kinase (SEK1) that acts as a SAPK activator but does not activate MAPK (36) . SEK is likely a physiological activator of SAPKs since it is activated by SAPK agonists and expression of kinase-dead forms of SEK block activation of SAPK in intact cells. The structural similarities between SAPK and MAPK and SEK and MEK suggests that an upstream kinase analogous to Raf would similarly phosphorylate and activate SEK. We report here that MEKK activates the SAPK pathway by direct phosphorylation and activation of SEK, leading to activation of SAPK. Moreover, although MEKK functions *in vitro* and in overexpression systems to activate MAPK, we show that stable cell lines expressing regulated MEKK fail to activate MAPK at levels of expression that strongly stimulate

SAPK. We conclude that MEKK is a natural activator of the stress activated kinase pathway, and has a secondary role in activating MAPK.

Methods

Cell Culture and gene expression.

Kinases were expressed in CV1 monkey kidney cells (ATCC) maintained in DME with 10% calf serum using the plasmid vector pTM1(12, 13) , which encodes protein products under the control of the bacteriophage T7 promoter. To express the proteins in eukaryotic cells, the plasmid-transfected cells were infected with the recombinant vaccinia virus vector vTF7-3, that encodes the T7 RNA polymerase, and then were transfected with the plasmid vector. Transfection and infection of CV1 cells using this system has been described previously (54) .

Plasmid constructions

Rat MEK-1 (2) and mouse MEKK (10) were cloned by RT-PCR using primers designed from the published sequence. The coding regions were expressed as an epitope tagged protein, with a synthetic amino-terminal epitope (EEEEYMPME, termed "EE") derived from middle T antigen of polyomavirus. This antibody, and suggestions for its use were from Gernot Walter, UCSD. In this paper, we have used exclusively a truncated active fragment of MEKK, representing a deletion of the amino terminal 367 amino acids, termed ΔMEKK. A truncated, active form of c-raf, in which the 303 amino-terminal amino acids is deleted, was also used in these experiments (termed Δraf) and was also epitope tagged with the EE epitope. A cDNA encoding murine MAP kinase (p42) was obtained from Michael Weber (University of Virginia) and was modified by addition of an amino terminal EE epitope as for the other kinases. Inactive "KR" mutations used were, for Δraf— K375R, for ΔMEKK— K447R; for MEK-1— K97R, and for MAPK— K46R.

Mutagenesis.

A variation of the "megaprimer" PCR method of mutagenesis ADDIN (55) ADDIN was used. Small restriction fragments containing the mutagenized region were subcloned into unmutagenized plasmids, and the entire mutagenized region sequenced to assure against unexpected mutations.

Immunopurification of kinases.

Transfected cells expressing epitope tagged kinaseswere lysed in MLB (50 mM MOPS•Na pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1 % NP40 and 1 mM DTT, containing the protease inhibitors aprotinin (2.5μg/ml) leupeptin (2.5 μg/ml) and PMSF (50 μg/ml) and the phosphatase inhibitors NaF (10 mM), Sodium Pyrophosphate (5 mM), Na_3VO_4 (1 mM) and β-glycerol phosphate (10 mM). After clarification of the cell extracts, epitope-tagged kinases were precipitated using Affigel 10 beads (BioRad) coupled to purified EE monoclonal antibody (3 mg antibody per ml gel). To purify protein from 10^7 transfected cells, 20 μl of this affinity matrix was used. Immune complexes were collected and washed in MLB by spin filtration using microfiltration columns ("Compact Reaction Columns", USB). Bound proteins were eluted overnight in 25 μl of elution buffer (50 mM Tris•Cl pH 7.4, 0.5 mM DTT, 10 mM β-glycero phosphate, 1 mM Na_3VO_4, 20% glycerol) including 40μg/ml EE peptide (N-Ac-EEEEYMPME-COOH), and the filtrate recovered by centrifugation. Typically, the resulting solution contained 50 to 200 ng kinase per μl, and the immunopurified kinase is the only protein detected using Coomassie gel staining.

in vitro phosphorylation

Routinely, one μl of the recovered kinase from the procedure above was used to phosphorylate 4 μl of an identically-purified kinase substrate. The 20 μl reaction mixture contained 10 μM ATP,

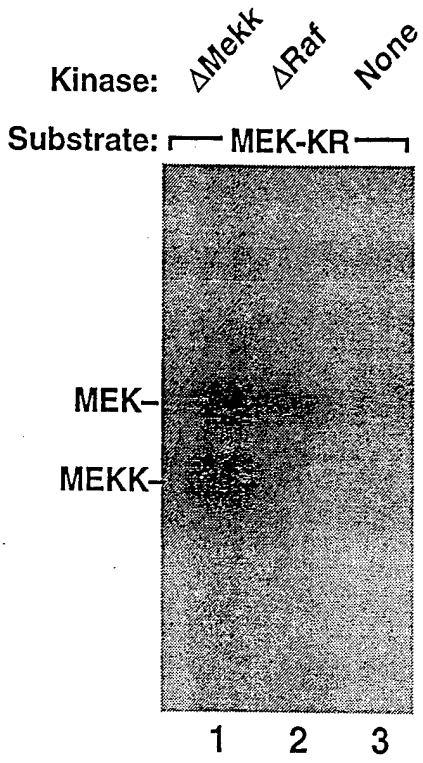

Figure 2. Phosphorylation of MEK-1 *in vitro.*

ΔMEKK and Δ*raf* were immunopurified as described in Experimental Procedures, and used to phosphorylate a kinase inactive form of MEK-1, termed MEK-KR. MEK-KR is strongly phosphorylated by both MEKK and *raf*, but is devoid of autophosphorylating activity, since it is not phosphorylated when incubated without the other kinases (lane 3). ΔMEKK is also strongly phosphorylated, and Δ*raf* is slightly phosphorylated, faintly visible in this experiment, migrating between ΔMEKK and MEK.

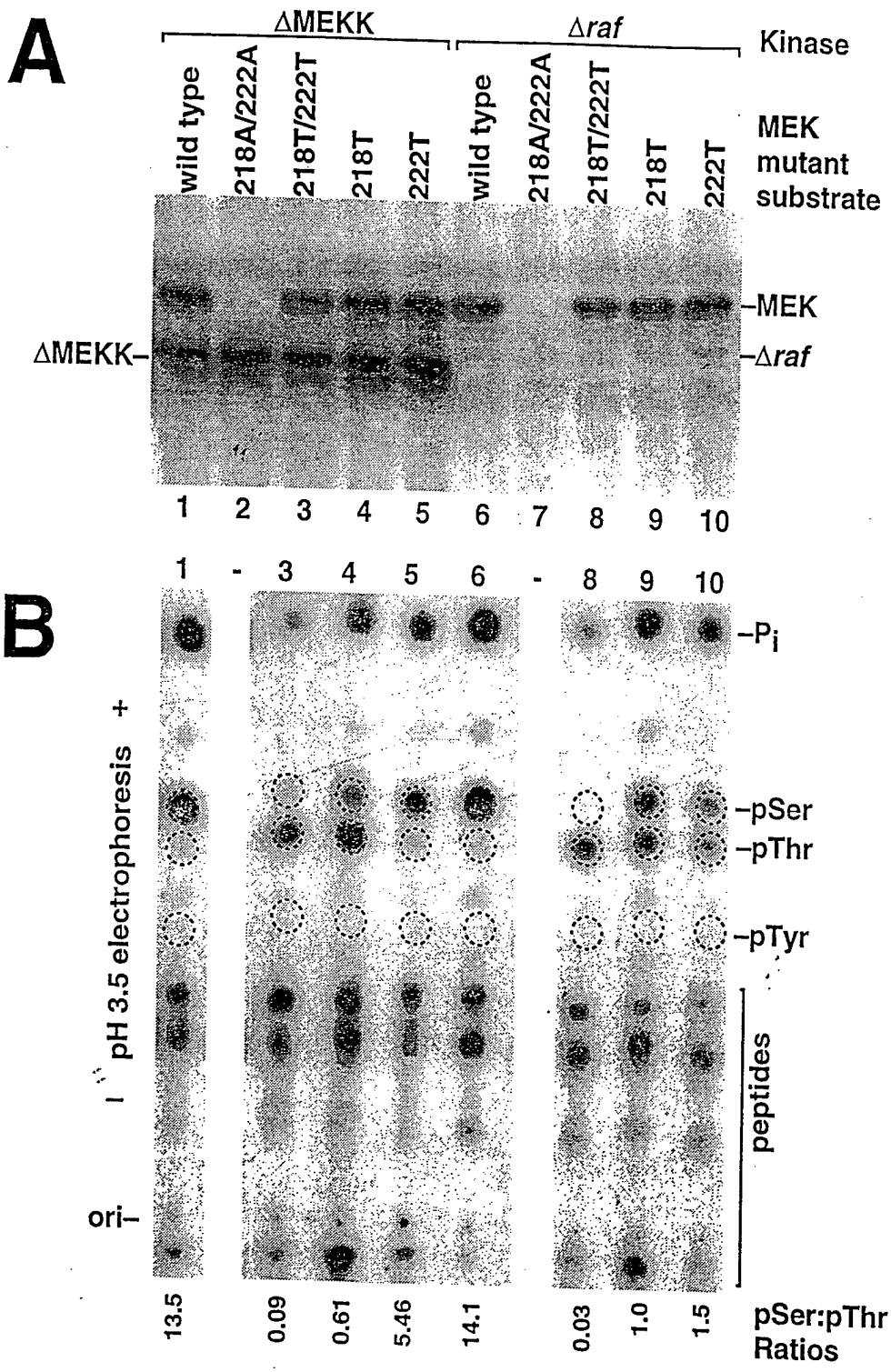
A
ΔMEKK
Δraf
Kinase
wild type
218A/222A
218T/222T
218T
222T
wild type
218A/222A
218T/222T
218T
222T
MEK mutant substrate
–MEK
–Δraf
ΔMEKK–
1 2 3 4 5 6 7 8 9 10
B
1 - 3 4 5 6 - 8 9 10
–Pi
–pSer
–pThr
–pTyr
peptides
+
pH 3.5 electrophoresis
–
ori
13.5
0.09
0.61
5.46
14.1
0.03
1.0
1.5
pSer:pThr Ratios

Figure 3. Identification of sites phosphorylated on MEK-1 by *raf* and MEKK.

Two MEK phosphorylation sites were identified as described in the text, and mutations of each site were made in which these sites were both altered to alanine residues or were changed singly or together to threonine residues. Each MEK protein also contained the inactivating K97 R mutation to prevent autophosphorylation.

Panel A. Phosphorylation of MEK mutants by *raf* or MEKK. Immunopurified MEK-1 or mutant proteins were incubated *in vitro* with immunopurified ΔMEKK (lanes 1 through 5) or with Δ*raf* (lanes 6 through 10) together with ^{32}P-labeled ATP. The reaction products were separated by SDS-PAGE followed by transfer to Immobilon membranes for autoradiography. All mutant MEK proteins were phosphorylated to approximately the same level, with the exception of the 218A/222A double mutant, which was not phosphorylated by either activating kinase. ΔMEKK was strongly autophosphorylated in this reaction, and Δ*raf* was weakly autophosphorylated.

Panel B. Phosphoamino acid analysis of *in vitro* phosphorylated MEK-1 mutants. Bands of radiolabeled MEK from the experiment shown in Panel A (Lanes 1, 3-6, and 8-10) were excised from the Immobilon membrane and subjected to acid hydrolysis (see Experimental Procedures). Resultant hydrolysates were separated by one-dimensional thin layer electrophoresis at pH 3.5. The position of unlabeled phosphoamino acid markers is indicated by the dotted circles, as is that of free phosphate and the partially hydrolyzed peptides. Both ΔMEKK and Δ*raf* are able to phosphorylate mutant threonine residues located at the position of the naturally-phosphorylated serines. No serine phosphorylation is seen in the 218T/222T mutant, demonstrating that no other residues of MEK are phosphorylated by these kinases *in vitro*. Relative levels of phosphoserine and phosphothreonine quantified from this experiment are shown beneath each lane.

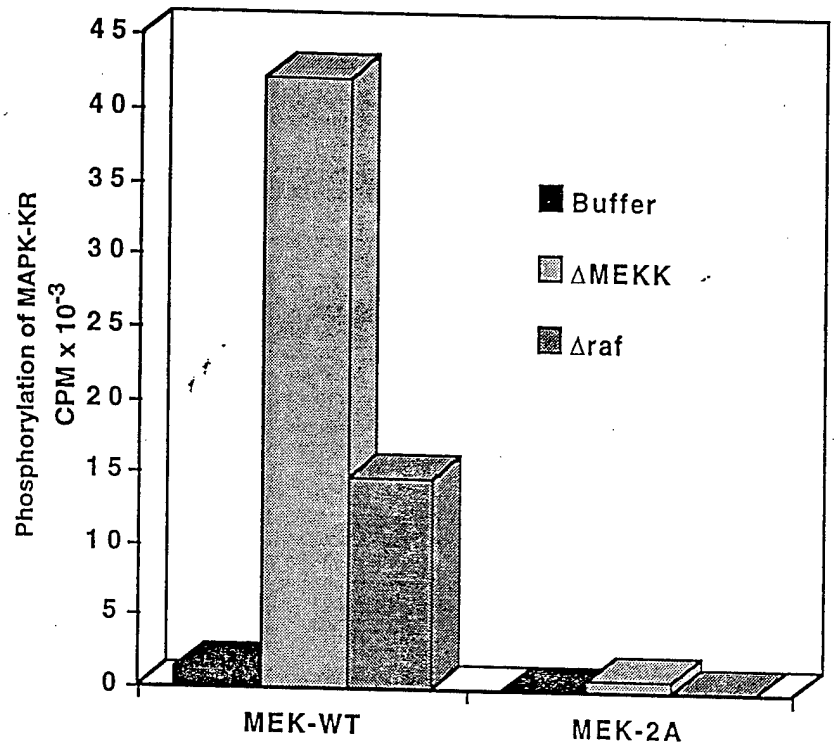

Figure 5. S218 and S222 are required for activation of MEK *in vitro* by MEKK or *raf*. Immunopurified MEK-WT, or MEK S218A/S222A mutant was activated *in vitro* with purified ΔMEKK, Δ*raf*, as described in Experimental Procedures, using MAPK-KR as substrate. After SDS-PAGE and autoradiography, labeled MAPK was excised from the gel and quantified by Cérenkov counting. MEK-WT but not the S218A/S222A mutant was efficiently activated by ΔMEKK and Δ*raf*.

10 μCi γ-^{32}P-ATP, in 50 mM Tris•Cl pH 7.4, 0.5 mM DTT, 10 mM $MgCl_2$. After 30 minutes at RT, reaction was stopped by the addition of SDS/DTT sample buffer and boiling. Because we found that the epitope-tagged Δ*raf* kinase was only fractionally eluted from the beads using this procedure, in some reactions (those in Figure 7) reactions were performed using kinases that remained attached to the immune affinity beads. Kinase reaction conditions were otherwise as shown above.

Phosphoamino acid analysis

Phosphoproteins blotted onto Immobilon were hydrolyzed in 6 M HCl at 110°C for one hour according to Kamps (56) and analyzed by thin layer electrophoresis at pH 3.5.

Inducible MEKK1 expression.

The kinase active carboxyl-terminal 320 amino acids of MEKK1 was expressed in clones of NIH3T3 cells using the lac regulated promoter lacSwitch (Stratagene). MEKK was cloned and epitope tagged using the EE epitope (MHEEEEYMPME) as described previously Individual clones of cells were induced in 1 mM IPTG and tested by immunoprecipitation of epitope tagged protein from cell extracts using the anti-EE monoclonal antibody, followed by detection of the epitope tagged MEKK by SDS-PAGE and immunoblotting using the same antibody.

Results

MEKK expression activates SAPK not MAPK

In experiments designed to investigate signaling downstream of raf and MEKK, we undertook to isolate cell lines that express these MAPK activators in a regulated fashion. Early in the course of these experiments it became obvious that unlike raf, MEKK exerted growth inhibitory effects when expressed in tissue culture cells. To this end, clones of NIH3T3 cells were isolated that express MEKK in response to IPTG, using an engineered promoter controlled by the *lac* repressor. Several cell lines were obtained, which shared the characteristic of MEKK expression coupled with growth inhibition in response to IPTG stimulation (Figure 9). Surprisingly, while active MEK Kinase activity was found within the cell, the level of MAPK activity was unchanged in the MEKK induced cells (see Figure 10, below).

One explanation for this discrepancy was that the effects of MEKK expression reflected activation of a signaling cascade distinct from MAPK in these cells. Since MEKK expression resulted in growth inhibition, we tested whether activation of the SAPK pathway, also associated with growth inhibitory signaling, was activated by MEKK in these cells. We stimulated parent NIH3T3 cells or two independent lines of inducible MEKK cells with IPTG and measured MAPK and SAPK activities in parallel. As before, activity of MAPK (p42) in these cells was unchanged by MEKK expression (Figure 11A). In contrast, SAPK activity was six- to eight-fold elevated in the MEKK-inducible cell lines but was not elevated in the parent NIH3T3 cells treated with IPTG. These MEKK inducible cells are still able to activate MAPK in response to some mitogenic signals, since treatment with TPA resulted in increases in MAPK activity in each of these clones (as well as in NIH3T3 cells), while SAPK activity was unaffected (Figure 11B).

One of the inducible cell lines was additionally tested for SAPK and MAPK activity at varying time points after MEKK induction (Figure 11C). Increased SAPK activity was evident by 3 hours and became maximal after 12 hours of induction, while MAPK activities remained unchanged throughout the 23 hour incubation with inducer. Together, these results suggest that in contrast to the previously supposed role of MEKK in activating MEK and MAPK, it acts instead to activate SAPKs.

Reconstitution of the MEKK-SEK-SAPK cascade.

Since elevations of SAPK activity in response to MEKK expression might reflect global, and possibly indirect "stressful" changes within the cell, we sought to model activation of SAPK by MEKK

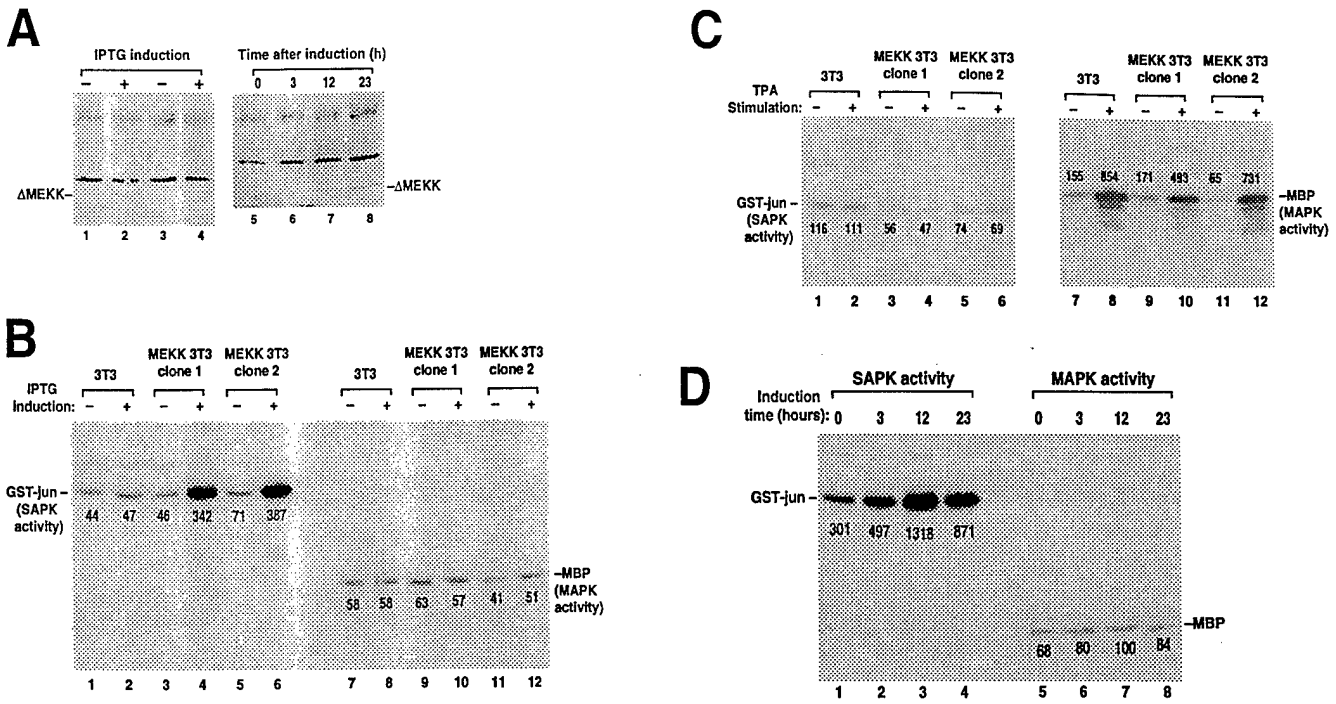

Figure 9.

A: MEKK expression in NIH3T3 cells. Epitope tagged truncated MEKK (ΔMEKK) was detected in MEKK clone 1 cells (lanes 1 and 2) or in MEKK clone 2 cells (lanes 5-8) but not in a control clone (lanes 3 and 4) treated with IPTG for 24 hours (lanes 2 and 4) or at the indicated times (lanes 6-8).

B: MAPK and SAPK activity in MEKK inducible cell lines. Numbers below the labeled bands indicate CPM of radioactivity in substrates. SAPK activity, but not MAPK activity was increased in response to MEKK expression.

C: Functional MAPK signaling demonstrated in NIH3T3 cells and MEKK expressing subclones clones after stimulation (+) with 250 ng/ml TPA.

D: Time course of induction of SAPK activity in MEKK 3T3 clone 2 cells after IPTG treatment. MAPK activity throughout this period remained unchanged, while SAPK activity was increased even at the 3 hour time point, when MEKK expression could not yet be detected. MEKK expression in cells used in this experiment is shown in Panel A, lanes 5-8.

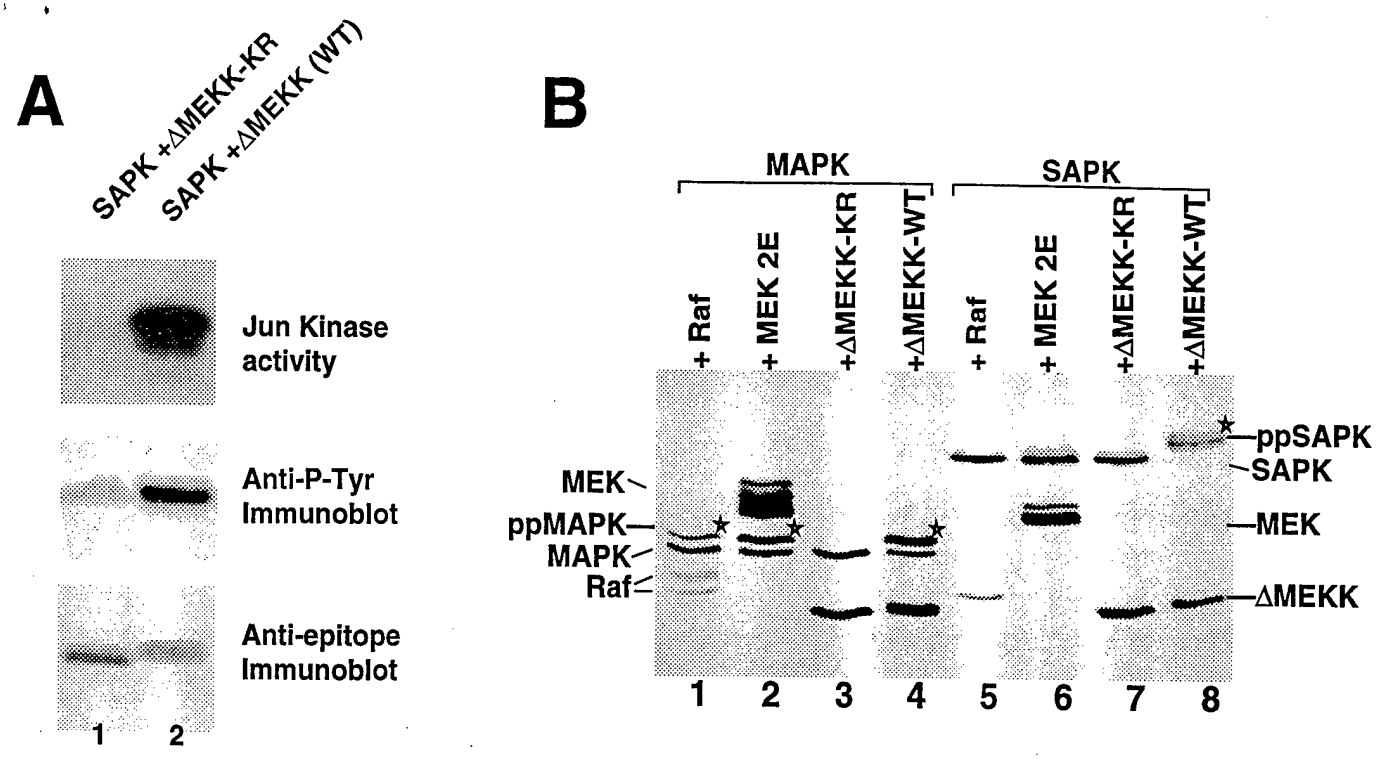

Figure 10

Activation of the SEK-SAPK pathway by coexpression of ΔMEKK using vaccinia virus vectors.

A: Epitope tagged SAPK was expressed with either the untagged, inactive KR mutant of ΔMEKK (lane 1), or wild type ΔMEKK (lane 2). Co-expression of active MEKK resulted in mobility shift of SAPK detected by anti-epitope immunoblotting (bottom panel) and also increased tyrosine phosphorylation of SAPK detected in anti-epitope immunoprecipitates (middle panel). SAPK activity was also strongly elevated, reflected by phosphorylation of GST-Jun(5-89) using anti-epitope immunoprecipitates (upper panel).

B: Epitope tagged MAPK (lanes 1-4) or SAPK (lanes 5-8) was expressed with epitope tagged forms of truncated active Raf (lanes 1, 5), constitutively active MEK 2E (lanes 2, 6), ΔMEKK-KR mutant (lanes 3, 7) or ΔMEKK wild type (lanes 4, 8), and detected in whole cell lysates using anti-epitope western blot. Activation of both MAPK and SAPK is identifiable by the appearance of bands with delayed mobility, indicated by stars. Raf and active MEK 2E are able to activate MAPK, but not SAPK, thus the SAPK pathway is insulated from the MAPK pathway. MEKK is able to activate MAPK in this overexpression system, though it is not when expressed at lower levels (Figure 1). Of the kinases tested, only MEKK is able to activate SAPK.

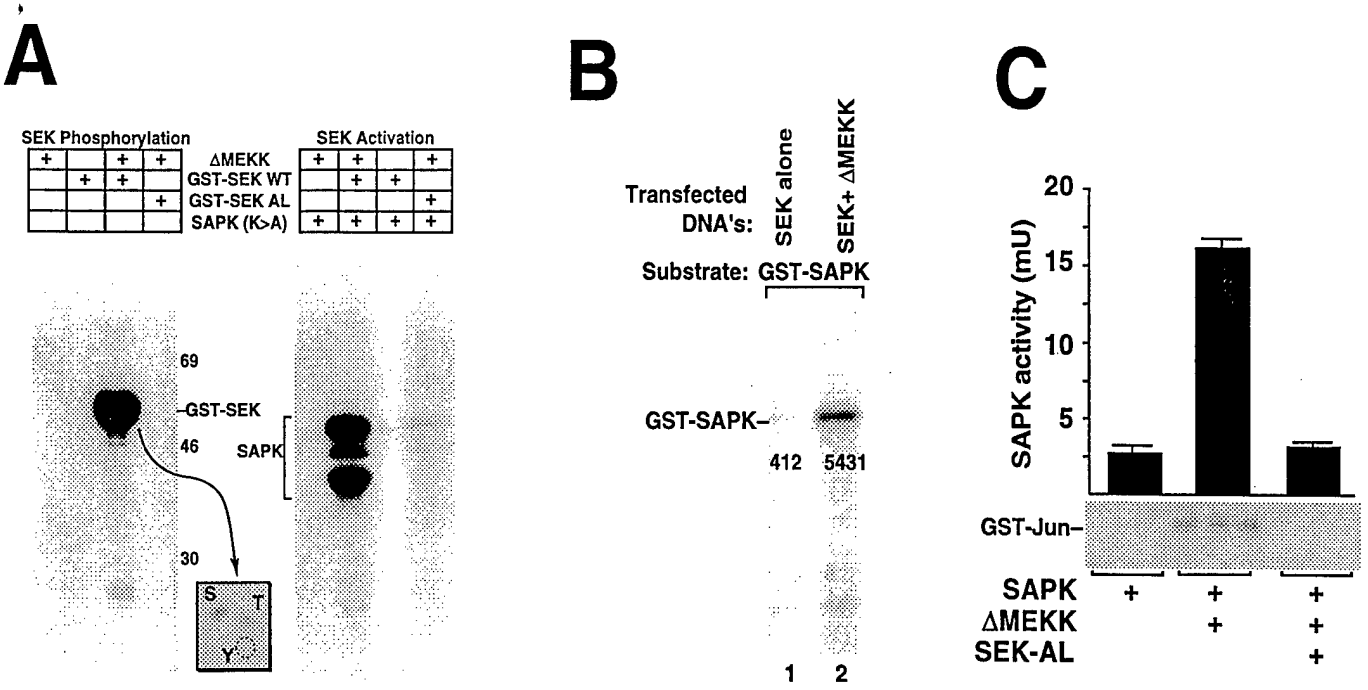

Figure 11.

A: MEKK Phosphorylates and activates of SEK *in vitro* . Immunopurified ΔMEKK phosphorylated wild type GST-SEK (lane 3) on serine and threonine (see phosphoaminoacid analysis, inset), but not mutant GST-SEK protein lacking the two phosphorylation sites (lane 4). GST-SEK1 phosphorylated by MEKK *in vitro* acquired SAPK kinase activity demonstrated after secondary reaction with inactive (K>A) mutant thrombin-cleaved GST- SAPK with radioactive ATP (lane 6). Mutant GST SEK protein lacking phosphorylation sites (lane 8) or reactions without either MEKK or SEK did not phosphorylate SAPK.

B: MEKK activates SEK1 *in vivo*. Epitope tagged SEK expressed in CV1 cells using a CMV expression vector (Lane 1) became activated by coexpression of ΔMEKK lacking the epitope tag. Anti-epitope immunocomplexes were assayed for SEK activity using GST-SAPK as substrate as for Figure 1.

C. SAPK activation by MEKK requires SEK1. Epitope tagged SAPK expressed using SV40-based vectors was activated by coexpression with ΔMEKK. This activation was reversed by triple coexpression of a dominant inhibitory mutant of SEK1 containing (S220A, T224L; SEK AL).

using cloned genes and purified proteins. The proteins were expressed using a high level expression system using vaccinia virus vectors (13) that allow surveillance of epitope tagged kinases by immunoblotting, as well as purification by immunoaffinity chromatography and subsequent assays of kinase activities. We first expressed epitope tagged SAPK together with MEKK or a point mutant of MEKK containing an inactivating mutation of the ATP binding pocket of MEKK (mutant K375R). When expressed in conjunction with active MEKK, SAPK was found by immunoblotting to be electrophoretically retarded which was suggestive of quantitative phosphorylation (Figure 12). Immunoblotting of these cell lysates using anti-phosphotyrosine monoclonal antibodies revealed a large increase in the amount of phosphotyrosine associated with SAPK. Lastly, the kinase activity of the epitope tagged SAPK was measured *in vitro*, and found to be increased many fold by coexpression with MEKK, but not by expression with the inactive MEKK mutant. Thus, in this overexpression model as well as in the inducible cell line, MEKK expression results in activation of the SAPK pathway.

Since MEKK has been shown to be able to activate MAPK using this overexpression system (but does not activate MAPK in the MEKK inducible cell lines described above) we considered the possibility that activation of SAPK occurred as a consequence of the MEK and MAPK cascade. In order to stimulate MAPK independently of MEKK, we expressed SAPK together with activated raf, or with a constitutively active allele of MEK containing glutamic acid residues at the sites of activating phosphorylation (termed MEK 2E, see Part One). Both raf and MEK 2E were able to induce phosphorylation of coexpressed MAPK (Figure 10). However, neither of these MAPK activators induced phosphorylation of SAPK. This result indicates that the SAPK activation pathway is effectively insulated from the MAPK pathway, and that activation of SAPK in response to MEKK is unlikely to result through intermediate activation of MAPK.

Using immunopurified MEKK and SAPK proteins, we found that MEKK was unable to phosphorylate SAPK *in vitro* (not shown). We therefore tested whether the newly-identified SAPK activator, SEK1 served as an intermediate in the activation of SAPK by MEKK. Immunopurified MEKK (but not the kinase inactive mutant of MEKK) rapidly phosphorylated a GST-SEK fusion protein but failed to phosphorylate a SEK mutant at which the two residues analogous to the sites of activation in MEK were mutated (Figure 6). Moreover, phosphorylation of GST-SEK activated the SAPK kinase activity of this molecule. Thus, SEK is a direct substrate of MEKK and activation of SEK by MEKK is sufficient to activate SAPK.

A signaling cascade leading to growth inhibition in mammalian cells

These results demonstrate complete reconstitution of a kinase cascade, beginning with MEKK, that phosphorylates and activates SEK, which subsequently phosphorylates and activates SAPK. Each component of this cascade is functionally parallel to a component of the MAPK activation pathway: SAPK is analogous to MAPK, SEK analogous to MEK, and MEKK analogous to raf. Given this observation, a proper term for MEK Kinase might instead be "SEK Kinase" or SEKK.

Activation of the SAPK pathway is separable from that of MAPK. As we show here, activation of MAPK either by raf or by constitutively active alleles of MEK are unable to induce activation of SAPK. Conversely, cell lines expressing MEKK have elevated SAPK but do not have elevated MAPK; therefore, there appears to be little if any crosstalk between the two pathways downstream of MEK. Is MEKK able to activate MEK, as original proposed? Clearly, when tested *in vitro*, MEKK is able to phosphorylate and activate MEK. MEKK is also able to activate MEK (and subsequently MAPK) when overexpressed in COS-7 cells (10) . Our group has recently confirmed MEKK phosphorylation of MEK *in vitro* and when using the vaccinia virus expression system (and see Figure 10), but found that unlike raf, MEKK demonstrates marked phosphorylation site preference for one of two activating sites of MEK (codon S222 over S218), thus even *in vitro* MEKK and raf are not functionally identical. Additionally, genetic evidence argues that MEKK plays a minor, if any, role in MEK activation, since

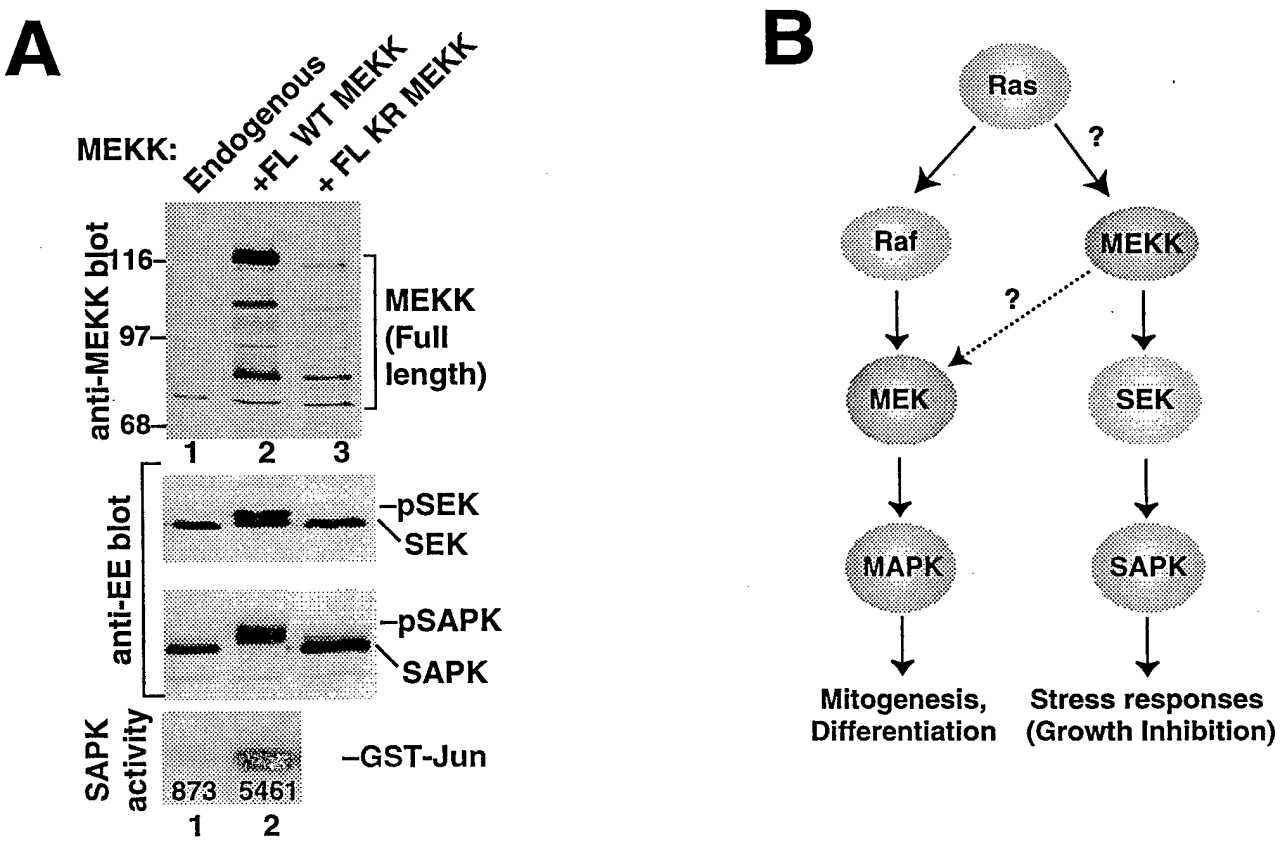

Figure 12.

A. Activation of SEK and SAPK *in vivo* by full length MEKK.

Using the vaccinia virus expression system in CV1 cells, SEK1 or SAPK were separately expressed alone (Lane 1) or together with vectors encoding full length wild type MEKK1 (Lane 2) or a kinase inactive (K447R) mutant allele of MEKK1 (Lane 3). Expression of several bands related to full length MEKK (top panel) was detected using chicken polyclonal antibodies raised against bacterially expressed ΔMEKK. Endogenous proteins of 70kd, 90kd, and 110kd were also detected (Lane 1). Epitope tagged SEK1 and SAPK1(middle panels) were detected by immunoblotting. Electrophoretically retarded bands arising from phosphorylation of both SEK and SAPK were observed from coexpression with full length MEKK, indicating that MEKK thus expressed is constitutively active. SAPK activity was activated by full length MEKK (bottom panel).

B: Diagram of separate pathways emanating from Raf and MEKK. Both Raf and MEKK appear to be dependent upon the function of Ras, as described in the text. The Raf-MEK-MAPK pathway is functionally analogous to the MEKK-SEK-SAPK pathway, although the result of stimulation of each pathway is distinct. A dotted arrow from MEKK to MEK reflects the ability of MEKK to phosphorylate MEK *in vitro* and during high level cell expression. However in stable inducible cell lines, MAPK activation by MEKK is not seen, drawing the physiological significance of this path into question. The opposing nature of two signaling pathways both emanating from Ras suggests an important role for factors cooperating with Ras to provide specificity for stimulation of one path versus the other.

mutant Drosophila MEK rescues most of the functions ablated in D-Raf null mutants in both the Torso and R7 photoreceptor pathways (37). This implicates Raf as the major physiological MEK activator.

In vivo, in the stable MEKK inducible NIH3T3 cells studied here, the answer is clear: MEKK activity activates SAPK and not MAPK, even though these cells express an intact MAPK activation pathway. Possibly, MEKK might stimulate MAPK in other cell types, or perhaps it has a transient role in activating MAPK, although this was not apparent in our time course experiment that monitored MAPK activity as early as 3 hours, at which time SAPK activity was elevated and MEKK protein expression was below detectable levels.

Perhaps the appropriate view of MEKK signaling would be as an *analog* instead of a *binary* cell regulator. Given this view, depending on the interplay between kinase activity, substrate availability, and the intracellular milieu, MEKK might activate SAPK within our experimental systems, while other cellular circumstances might translate MEKK activity into a variety of mixed signals including SAPK.

Analysis

Signaling through MEKK, SEK, and SAPK

The nature of signals that lead to activation of SAPK is still unresolved, but attention can now be placed on signaling that might pass through MEKK. SAPK is known to be activated in response to UV light, cycloheximide, anisomycin, and TNF-α; these agents potentially mediate activation of MEKK within cells as well. Alternatively, many or all of these agents could bypass MEKK and activate SAPK via action of alternative kinases.

Potentially, MEKK could be activated by phosphorylation, as apparently can raf (38, 39). We have identified sites of phosphorylation of MEKK that are required for activity (J. C. D. and D. J. T., in preparation); phosphorylation of these sites could be altered in response to TNF-α, UV irradiation or anisomycin treatment. TNF-α treatment of cells induces activity of a membrane associated protein kinase using the second messenger ceramide (40); MEKK could represent a subsequent target of activation by this kinase.

MEKK could also be activated via direct interaction with some mediating protein, perhaps the ras oncoprotein. This possibility is naturally attractive because of the obvious parallel between MEKK and the c-raf protein, which is activated by binding to ras. Similar to c-raf, MEKK naturally encodes a large amino terminal section that represents a potential regulatory domain. Interaction between MEKK and ras, the TNF-α receptor, or other proteins via this domain could serve to transmit signals through MEKK.

Other lines of evidence suggest that MEKK-SEK-SAPK signaling lies downstream of ras. First, activation of SAPK in response to UV light is potentiated by oncogenic ras (34) , and blocked by dominant inhibitory mutants of ras (41). Secondly, expression of the dominant inhibitory N17 allele of ras reduces the activity of MEKK (42). Thirdly, cell death in response to TNF-α is strongly enhanced by expression of oncogenic ras, without increase in TNF-α receptor expression (43). Lastly, association of the MEKK homolog byr2 and ras1 in yeast has been detected using a two-hybrid strategy (9). Thus, in parallel to c-raf, MEKK and the stress pathway it activates most likely lies within a pathway downstream of ras.

This observations raises a possible contradiction, however: SAPK and MAPK pathways are separately and distinctly activated by different effectors (e.g. EGF for MAPK and TNF-α for SAPK), and the effects of these signals on cells are contrasting (mitogenesis versus growth arrest), yet activation of both pathways is dependent on ras. To answer this, cofactors for ras must exist that contribute specificity to each pathway. Clearly, characterized proteins that cooperate with ras, such as GAP, GRB2, or mSOS (or related proteins), or the stimulating factor receptors themselves, could provide a

mechanism by which specificity of signaling is maintained. Since activity of the src oncoprotein has been shown to contribute to ras-dependent signaling through raf (44), members of the tyrosine kinase family could represent such cofactors leading to activation of the MAPK, but not necessarily the SAPK pathway. Cooperative factors leading to MEKK signaling have not been described, and their identification is a major objective.

The best candidate for a physiologically relevant substrate of SAPK is c-Jun. Phosphorylation of c-jun in response to cellular stressors is well documented and was confirmed in response to MEKK in our expression system (Figure 3). Phosphorylation of c-Jun at the amino terminal sites recognized by SAPK is required for transactivation (32).

Other potential SAPK signaling targets could represent molecules involved in growth arrest in response to UV irradiation or TNF-α treatment. The p53 tumor suppressor protein is more abundant in cells following UV irradiation, possibly resulting from stabilization. p53 has been reported to be phosphorylated by MAPK at two amino terminal serines (45), and phosphorylation of these sites is increased after UV exposure. These sites could be a target of SAPK as well.

In the yeast pheromone response pathway, G1 cell cycle arrest is dependent upon phosphorylation of the nuclear protein Far1, which binds to and inhibits the kinase activity of Cdc28 associated with Cln (46, 47). Exact mammalian homologs of Far1 are not determined, but several nuclear proteins have been identified that inhibit CDK/cyclin complexes by similar mechanisms (48, 49, 50, 51, 52, 53). Since MEKK is a structural homolog (10), of signaling kinases of yeast (Byr2/Ste11) upstream of Far1, a logical possibility would be that signaling by MEKK through SAPK could result in growth inhibition by activation of a cell cycle inhibitor similar to Far1.

Here we have identified another component of a pathway via which cells respond to stress. Furthermore, activation of this pathway induces cell growth inhibition. Together these observations should facilitate identification of the remaining signaling molecules leading from the plasma membrane to nuclear growth control molecules.

CONCLUSION

This award was used to train Dr. Minhong Yan during his Ph.D. research, and as such has been highly successful. Dr. Yan received his Ph.D. in May of 1997, and began a Post-Doctoral fellowship in July 1 1997.

Original Aims of this application are as follows.

- 1) **To identify sites of phosphorylation on MEK by *raf* and MEKK.** Phosphorylation of these sites is required for activation of MEK and further propagation of the signal. We will test activating and inhibiting mutants of these sites for their consequences in growth control.

This Aim has been accomplished and is represented in our Article in JBC:

Yan, M. and D. J. Templeton (1994) *Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase.* J Biol Chem **269:** 19067-19073.

This article is presented in the Appendix.

- 2) **To identify sites of phosphorylation on MEK and *raf* by MAP kinase.** Such "feedback" phosphorylation events may result in autoregulation of MAP kinase activity via an autoregulatory loop. We will attempt to modify this loop via genetic modification of the phosphorylation sites.

This aim was published by another group before funding was achieved:

Brunet, A., G. Pages, and J. Pouyssegur (1994) *Growth factor-stimulated MAP kinase induces rapid retrophosphorylation and inhibition of MAP kinase kinase (MEK1).* Febs Lett **346:** 299-303.

We have not pursued this topic further.

- 3) **To test MEKK activation following truncation of a regulatory domain, and to measure the ability of MEKK to function as an oncogene.**

This Aim was achieved as part of an extended project resulting in our paper defining the MEKK1-SAPK cascade:

Yan, M., T. Dai, J. C. Deak, J. M. Kyriakis, L. I. Zon, J. R. Woodgett, and D. J. Templeton (1994) *Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1.* Nature **372:** 798-800.

This article is included in the appendix.

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Papers stemming from this grant, including collaborative projects:

Yan, M. and D. J. Templeton (1994) *Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase.* J Biol Chem **269:** 19067-19073.

Yan, M., T. Dai, J. C. Deak, J. M. Kyriakis, L. I. Zon, J. R. Woodgett, and D. J. Templeton (1994) *Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1.* Nature **372:** 798-800.

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PERSONNEL

Only Dr. Minhong Yan was supported by this award.

APPENDICES:

ITEM 1 - REPRINT

Yan, M. and D. J. Templeton (1994) *Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase.* J Biol Chem **269**: 19067-19073

ITEM 2 - REPRINT

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Identification of 2 Serine Residues of MEK-1 That Are Differentially Phosphorylated during Activation by *raf* and MEK Kinase*

(Received for publication, April 26, 1994)

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The signal transduction kinase MEK (mitogen-activated protein (MAP) or extracellular signal-regulated (Erk) kinase)-1 is activated via phosphorylation by MEKK (MEK kinase) and *raf* kinases. We show here that these two kinases phosphorylate rat MEK-1 exclusively on two serine codons, Ser^{218} and Ser^{222}. Phosphorylation of MEK-1 on serines 218 and 222 is both necessary and sufficient for MEK-1 to be activated and able to phosphorylate MAP kinase. A mutant form of MEK-1 that replaces these two codons with alanine cannot be activated, and one that substitutes glutamic acid residues in place of these 2 serines is active independent of activation by phosphorylation. These sites of activation occur in a region of MEK-1 that is similar to sites of activating phosphorylation in several other serine/threonine kinases, suggesting that this region may represent a conserved "activating domain" of many kinases. MEKK and *raf* display differences in site preference between these two codons, with MEKK showing preference for the amino acid at codon 218 and *raf* phosphorylating each residue approximately equally. This site preference might result in differences in the temporal or subsequent substrate patterns of MEK activation that result from these two activation pathways.

Cell growth signal transduction is frequently accompanied by phosphorylation and activation of MAP[1] kinase, as reviewed recently (1). Two kinases capable of activating MAP kinase have been cloned, one termed MEK-1 (MAP or Erk kinase; alternatively MAP kinase kinase-1) (2, 3) and the other MAP kinase kinase-2 (4). MEK-1 is in turn activated by phosphorylation by the v-*raf* oncoprotein (5). Activation of c-*raf* is stimulated by the *ras* activation pathway, and recently direct interaction of c-*raf* with the *ras* oncoprotein has been demonstrated (6–9).

A kinase structurally unrelated to *raf* is similarly able to phosphorylate and activate MEK-1. This kinase, termed MEK kinase (or MEKK) because it lies immediately upstream of MEK, has recently been cloned (10), and several groups have characterized the enzymatic activity (see Ref. 11 for review). Although the growth signal that passes through MEKK might originate separately from the signal that passes through *raf*, the net effect, *i.e.* activation of MAP kinase, seems equivalent (10).

Since MEK-1 lies at the convergence of signaling pathways that may have distinct origins, the molecular details of MEK regulation are important. We have investigated the nature of MEK-1 activation by *raf* and MEKK and have developed *in vivo* and *in vitro* systems to model the activation of MEK. Using these model systems, we have identified the amino acid residues upon which *raf* and MEKK phosphorylate MEK-1 and show here that these sites are necessary for activation of MEK-1 *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Cell Culture and Gene Expression—Kinases were expressed in CV1 monkey kidney cells (ATCC) maintained in Dulbecco's modified Eagle's medium with 10% calf serum using the plasmid vector pTM1 (12, 13), which encodes protein products under the control of the bacteriophage T7 promoter. To express the proteins in eukaryotic cells, the plasmid-transfected cells were infected with the recombinant vaccinia virus vector vTF7-3 that encodes the T7 RNA polymerase and then were transfected with the plasmid vector. Transfection and infection of CV1 cells using this system has been described previously (14).

Plasmid Constructions—Rat MEK-1 (2) and mouse MEKK (10) were cloned by reverse transcriptase-polymerase chain reaction using primers designed from the published sequence. The coding regions were expressed as an epitope-tagged protein, with a synthetic amino-terminal epitope (EEEEYMPME, termed "EE") derived from middle T antigen of polyomavirus. This antibody, and suggestions for its use, were from Gernot Walter, University of California, San Diego. In this paper, we have used exclusively a truncated active fragment of MEKK, representing a deletion of the amino-terminal 367 amino acids, termed ΔMEKK. A truncated active form of c-*raf*, in which the 303 amino-terminal amino acids are deleted, was also used in these experiments (termed Δ*raf*) and was also epitope tagged with the EE epitope. A cDNA encoding murine MAP kinase (p42) was obtained from Michael Weber (University of Virginia) and was modified by addition of an amino-terminal EE epitope as for the other kinases. Inactive "KR" mutations used were, for Δ*raf*, K375R; for ΔMEKK, K447R; for MEK-1, K97R; and for MAPK, K46R.

Mutagenesis—A variation of the "megaprimer" PCR method of mutagenesis (15) was used. Small restriction fragments containing the mutagenized region were subcloned into unmutagenized plasmids and the entire mutagenized region sequenced to assure against unexpected mutations.

Immunopurification of Kinases—Transfected cells expressing epitope tagged kinases were lysed in MLB (50 mM MOPS·Na, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, and 1 mM dithiothreitol, containing the protease inhibitors aprotinin (2.5 μg/ml), leupeptin (2.5 μg/ml), and phenylmethylsulfonyl fluoride (50 μg/ml) and the phosphatase inhibitors NaF (10 mM), sodium pyrophosphate (5 mM), Na_3VO_4 (1 mM), and β-glycerol phosphate (10 mM). After clarification of the cell extracts, epitope-tagged kinases were precipitated using Affi-Gel 10 beads (Bio-Rad) coupled to purified EE monoclonal antibody (3 mg of antibody/ml of gel). To purify protein from 10^7 transfected cells, 20 μl of this affinity matrix was used. Immune complexes were collected and washed in MLB by spin filtration using microfiltration columns ("Compact Reaction Columns," U. S. Biochemical Corp.). Bound proteins were eluted overnight in 25 μl of elution buffer (50 mM Tris·Cl, pH 7.4, 0.5 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 20% glycerol), including 40 μg/ml EE peptide (*N*-Ac-EEEEYMPME-COOH), and the filtrate recovered by centrifugation. Typically, the resulting solution contained 50–200 ng of kinase/μl, and the immunopurified kinase is the only protein detected using Coomassie gel staining.

In Vitro Phosphorylation—Routinely, 1 μl of the recovered kinase from the procedure above was used to phosphorylate 4 μl of an identically purified kinase substrate. The 20-μl reaction mixture contained 10

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[1] The abbreviations used are: MAP, mitogen-associated protein; MEK, MAP or extracellular signal-regulated (Erk) kinase; MEKK, MEK kinase; MAPK, MAP kinase; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

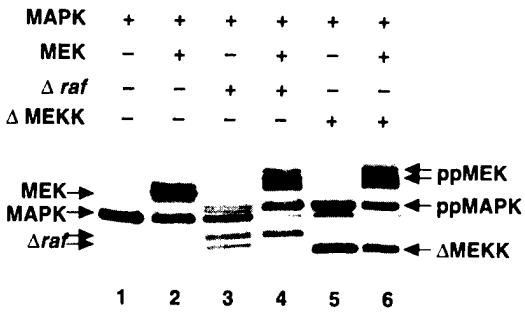

FIG. 1. ***In vivo* modeling of MAPK and MEK-1 activation.** MAPK was expressed in CV1 cells together with MEK-1 with or without activated forms of MEKK or *raf* as indicated. Each of the kinases were tagged with an amino-terminal synthetic epitope ("EE"), and the expression of each protein was monitored using anti-EE antibody for immunoblotting. MAPK expressed alone (*lane 1*) is minimally phosphorylated, as evidenced by the absence of the slowly migrating phosphorylated form. Co-expression of MEK with MAPK (*lane 2*) is insufficient to increase MAPK phosphorylation, unless an activating kinase such as *raf* (*lanes 3* and *4*) or MEKK (*lanes 5* and *6*) is included. In this case, MEK displays several forms with reduced mobility consistent with phosphorylation. MAPK is phosphorylated in response to the *raf* or MEKK activators, even more so in conjunction with MEK-1. Additionally, Δ*raf* shows a slowly migrating phosphorylated band, especially when expressed together with MEK (*lane 4*).

μM ATP, 10 μCi of [γ-^{32}P]ATP, in 50 mM Tris·Cl, pH 7.4, 0.5 mM dithiothreitol, 10 mM $MgCl_2$. After 30 min at room temperature, reaction was stopped by the addition of SDS/dithiothreitol sample buffer and boiling. Because we found that the epitope-tagged Δ*raf* kinase was only fractionally eluted from the beads using this procedure, in some reactions (those in Fig. 7) reactions were performed using kinases that remained attached to the immune affinity beads. Kinase reaction conditions were otherwise as shown above.

Phosphoamino Acid Analysis—Phosphoproteins blotted onto Immobilon were hydrolyzed in 6 M HCl at 110 °C for 1 h according to Kamps (16) and analyzed by thin layer electrophoresis at pH 3.5.

RESULTS

Expression of Signal Transduction Kinases—We used the T7-polymerase gene expression system of Moss (12, 13) to express MAP kinase (p42), MEK-1, and truncated kinase-active versions of MEKK and *raf*, either alone or in combinations within cells. To monitor expression of these protein kinases, and to afford simple means of micropurification of the wild type or mutant forms, we modified each of the proteins by the addition of a 10-amino acid synthetic epitope to the amino terminus of each protein. Each of the kinases is thus identifiable by Western blotting against the synthetic epitope (termed EE) and can be immunopurified under nondenaturing conditions.

When expressed alone using this system, MAPK is almost completely unphosphorylated (as shown in Fig. 1, *lane 1*), and phosphorylation of MAPK is unaffected by co-expression of MEK-1 (*lane 2*). This is probably because MEK itself is dependent upon upstream activation. When MAPK is expressed together with either Δ*raf* or ΔMEKK, increased phosphorylation of MAPK is seen, evidenced by a band with reduced electrophoretic mobility. Inclusion of MEK with these kinases results in nearly complete phosphorylation of MAPK (*lanes 4* and *6*), apparently due to expression of a complete activation pathway. In these lanes, MEK also shows a reduced electrophoretic migration compared with when it is expressed alone. This change reflects phosphorylation of MEK on serine and threonine residues (17) possibly from phosphorylation by Δ*raf* or ΔMEKK or by retrograde phosphorylation of MEK by MAPK or other kinases.

In Vitro Phosphorylation of MEK-1 by MEKK and raf—Activation of MEK-1 was also modeled *in vitro* using the active forms of MEKK or *raf* to phosphorylate MEK-1. To do this, we immunopurified epitope-tagged forms of both of these kinases

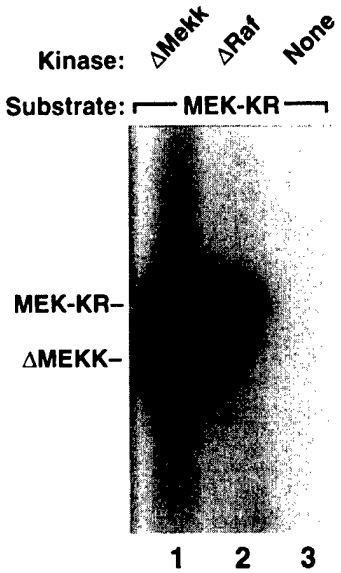

FIG. 2. **Phosphorylation of MEK-1 *in vitro*.** ΔMEKK and Δ*raf* were immunopurified as described under "Experimental Procedures" and used to phosphorylate a kinase inactive form of MEK-1, termed MEK-KR. MEK-KR is strongly phosphorylated by both MEKK and *raf*, but is devoid of autophosphorylating activity, since it is not phosphorylated when incubated without the other kinases (*lane 3*). ΔMEKK is also strongly phosphorylated, and Δ*raf* is slightly phosphorylated, faintly visible in this experiment, migrating between ΔMEKK and MEK.

(see "Experimental Procedures") and similarly purified a kinase inactive mutant of MEK-1 (containing a lysine to arginine mutation within the ATP binding domain of the kinase (18)). An example of this is shown in Fig. 2, in which both ΔMEKK and Δ*raf* are used to phosphorylate MEK-KR protein (*lanes 1* and *2*), whereas the preparation of substrate MEK-KR is itself completely devoid of phosphorylating activity (*lane 3*). ΔMEKK is also strongly phosphorylated, apparently through an autocatalytic reaction, whereas Δ*raf* is weakly phosphorylated (*lane 2*, but more clearly seen in Fig. 4). In experiments not shown, we found that MAPK phosphorylated MEK-1 on serine and threonine residues, whereas MEKK and *raf* phosphorylated exclusively serine residues. Since *raf* and MEKK are able to phosphorylate MEK-1 only on serine residues, and this phosphorylation was sufficient to activate MEK kinase activity (see Figs. 4 and 5 below), we focused on serine residues as potential sites of activating phosphorylation.

Identifying the Sites of MEK-1 Phosphorylation by raf and MEKK—Inspection of the predicted amino acid sequence of the rat MEK-1 protein reveals 18 serine residues, of which some are not conserved in MEK alleles or the homologous counterparts of MEK in other species. If activation of MEK-1 is conserved through its evolution, we predicted that the significant serine residues should either be identical in other alleles or possibly could be altered to threonine residues. Based on these suppositions, we initiated a program of directed mutagenesis targeting several serine codons, preparing these mutants in a background of the inactivated K96R MEK mutant to eliminate autophosphorylation by MEK.

Phosphorylation of mutant MEK proteins using ΔMEKK or Δ*raf* kinases showed that mutant S218A demonstrated reduced phosphorylation despite equivalent expression levels (not shown). However, mutation of this residue might have caused unpredictable changes in protein conformation, resulting in decreased phosphorylation at a distant site. To confirm phosphorylation of serine 218 genetically, we mutated this codon to a threonine residue. The level of phosphorylation of mutant protein S218T by ΔMEKK was equivalent to that of the wild

type protein. However, about 80% of the radiophosphate in this mutant protein was localized to threonine residues with the remainder on serine residues. Because a small amount of phosphoserine was still detected in mutant S218T, we surmised that at least one more site of serine phosphorylation was present on MEK.

We identified the additional phosphorylation site based on two observations. First, one of two chymotryptic peptides obtained during peptide mapping of the *in vitro* labeled MEK S218T mutant contained both phosphoserine and phosphothreonine (data not shown). This suggested that the two phosphorylation sites were contained within the same chymotryptic peptide. Second, the Ser^{218} residue lies within a domain similar to one in MAP kinase phosphorylated on two clustered residues (see Fig. 8). This lead us to speculate that MEK might be doubly phosphorylated in a similar cluster. We subsequently mutated each of the two serine residues near Ser^{218}, creating threonine codons, and found that only one of these mutant proteins (S222T) was phosphorylated on threonine. A double mutant in which both serine codons at 218 and 222 are altered to threonine contained no phosphoserine when phosphorylated by MEKK or *raf*. These experiments are shown in Fig. 3.

These data show that activated *raf* and MEKK phosphorylate the same 2 residues on MEK-1. MEKK shows apparent preference for the Ser^{218} residue *in vitro*, whereas *raf* phosphorylates each site roughly equivalently, based on the levels of phosphorylation of the individual threonine mutants. This observation is examined more closely below in Fig. 7. Additionally, the data in Fig. 3 show that no sites other than the serines at 218 and 222 are phosphorylated, since the 218T/222T mutant displays no phosphoserine, and the wild type protein displays no phosphorylation on threonine or tyrosine.

Do the Identified Sites Confer "Activatability" to MEK-1 in Vivo?—If Ser^{218} and Ser^{222} are required to activate MEK-1, an important prediction to test is that mutation of these sites would eliminate the ability of MEK to be activated by *raf* or MEKK *in vivo*. To test this prediction, we expressed epitope tagged MEK-1 proteins (wild type or alanine substitutions at the 218 and 222 sites, all EE-tagged) with or without active Δ*raf* proteins (without the EE epitope tag in this experiment). We then specifically immunopurified the epitope-tagged MEK proteins and assayed them for MAP kinase phosphorylation activity *in vitro*. As shown in Fig. 4, both of the single-site mutant proteins are still partly activated. Only the double alanine mutant demonstrated no activity either in the presence or absence of *raf*. Thus, both Ser^{218} and Ser^{222} contribute to the activatability of MEK by *raf*. The wild type MEK-1 protein was activated by co-expression with Δ*raf*, but clearly displays some basal activity even in the absence of co-transfected active *raf*. Since the plasmid-encoded proteins are expressed in the full context of normal cellular proteins, this low level of MEK activation almost certainly results from activation by uncharacterized cellular kinase(s), probably including but not exclusively the endogenous *raf* and MEKK kinases.

Do the Sites Confer Activatability in Vitro?—Since the activation of MEK-1 by *raf* in Fig. 4 occurred in intact cells, it remained possible that activation of MEK was not a result of *raf*, but rather by an intermediate *raf*-activated kinase. To model the activation of MEK *in vitro*, we purified *raf* and MEKK and used these kinases to phosphorylate MEK or the alanine-substituted mutant, in the presence of unlabeled ATP. Phosphorylated MEK protein was then incubated with an inactive mutant (K46R) of MAPK to quantify MEK activity. As shown in Fig. 5, both *raf* and MEKK are able to activate wild type MEK-1 *in vitro*, whereas the mutant substituting alanine residues at codons 218 and 222 is unable to be activated. Thus, these codons are required for direct activation by either *raf* or MEKK.

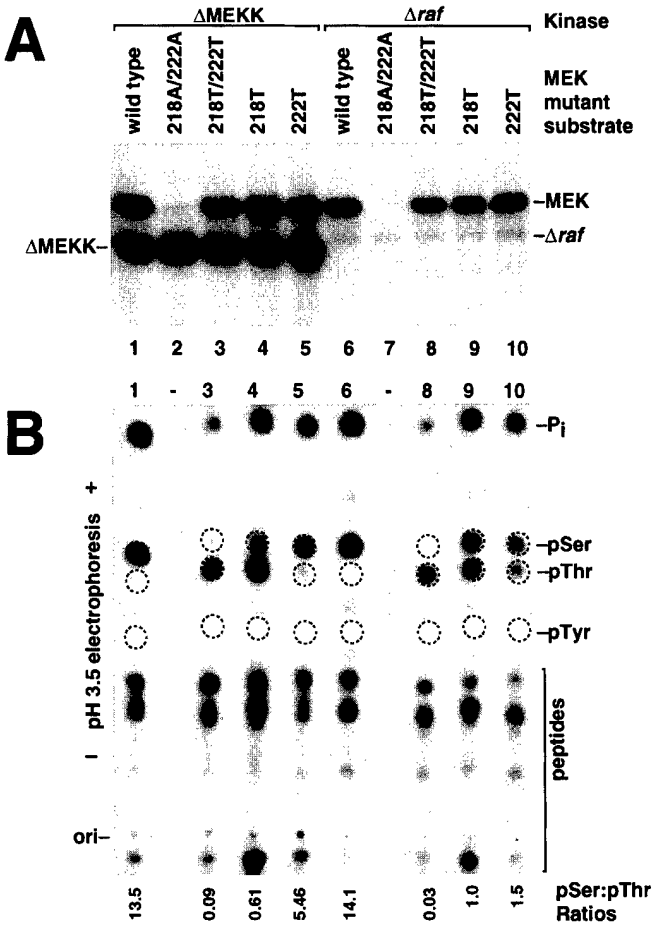

FIG. 3. **Identification of sites phosphorylated on MEK-1 by *raf* and MEKK.** Two MEK phosphorylation sites were identified as described in the text, and mutations of each site were made in which these sites were both altered to alanine residues or were changed singly or together to threonine residues. Each MEK protein also contained the inactivating K97R mutation to prevent autophosphorylation. *A*, phosphorylation of MEK mutants by *raf* or MEKK. Immunopurified MEK-1 or mutant proteins were incubated *in vitro* with immunopurified ΔMEKK (*lanes 1–5*) or with Δ*raf* (*lanes 6–10*) together with ^{32}P-labeled ATP. The reaction products were separated by SDS-PAGE followed by transfer to Immobilon membranes for autoradiography. All mutant MEK proteins were phosphorylated to approximately the same level, with the exception of the S218A/S222A double mutant, which was not phosphorylated by either activating kinase. ΔMEKK was strongly autophosphorylated in this reaction, and Δ*raf* was weakly autophosphorylated. *B*, phosphoamino acid analysis of *in vitro* phosphorylated MEK-1 mutants. Bands of radiolabeled MEK from the experiment shown in *A* (*lanes 1, 3–6,* and *8–10*) were excised from the Immobilon membrane and subjected to acid hydrolysis (see "Experimental Procedures"). Resultant hydrolysates were separated by one-dimensional thin layer electrophoresis at pH 3.5. The position of unlabeled phosphoamino acid markers is indicated by the *dotted circles*, as is that of free phosphate and the partially hydrolyzed peptides. Both ΔMEKK and Δ*raf* are able to phosphorylate mutant threonine residues located at the position of the naturally phosphorylated serines. No serine phosphorylation is seen in the 218T/222T mutant, demonstrating that no other residues of MEK are phosphorylated by these kinases *in vitro*. Relative levels of phosphoserine and phosphothreonine quantified from this experiment are shown beneath each lane.

Mutation of the Activation Sites to Glutamic Acid Results in Constitutive Activation—Since phosphorylation of MEK-1 at codons 218 and 222 introduces negative charges into this portion of the protein and results in kinase activation, we conjectured that substitution of negatively charged amino acids at these two positions might similarly activate a mutant MEK-1 protein. Such a constitutively active allele would be predicted

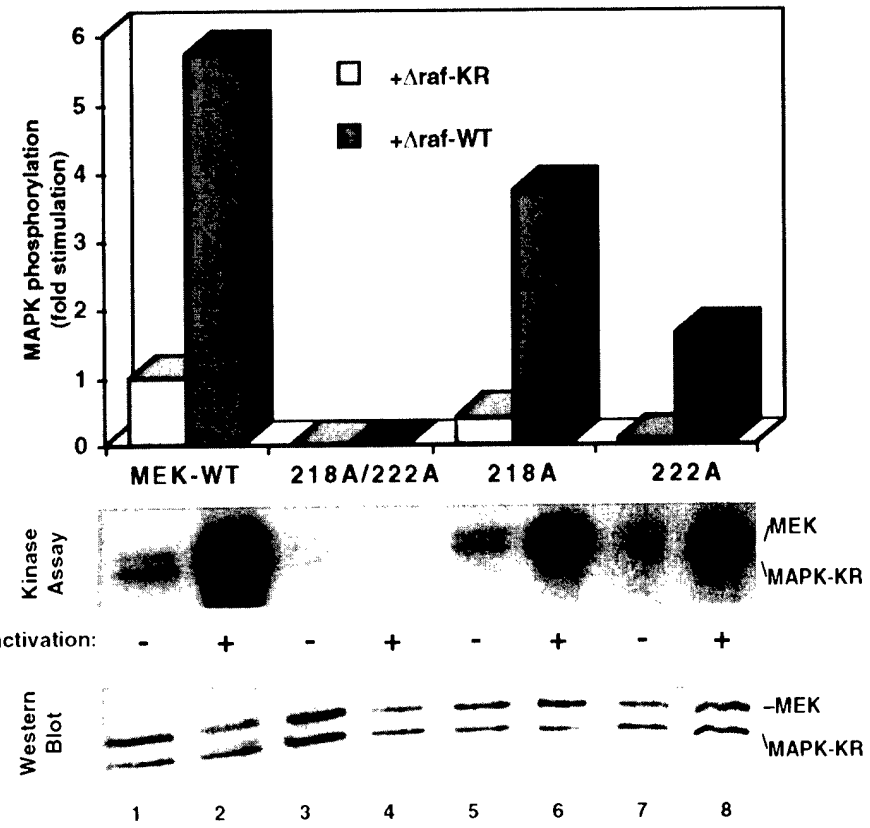

FIG. 4. **Ser^{218} and Ser^{222} are required for activation of MEK *in vivo*.** EE epitope-tagged MEK-WT, or alanine substitution mutants as indicated, were co-expressed with Δ*raf* or the inactive mutant Δ*raf*-K375R (that both lacked the EE epitope) to assess activation of MEK by Δ*raf*. EE-tagged MEK protein was specifically immunoprecipitated using anti-EE Affi-Gel beads and assayed for kinase activity by incubation with inactive epitope-tagged MAPK-KR substrate. Phosphorylated proteins were separated by SDS-PAGE and blotting onto Immobilon. Incorporation of radiophosphate into MAPK was quantified using an AMBIS β-imager and is shown in the *top panel*. The autoradiogram of this gel is shown in the *middle panel*. After quantification, the filter was probed using anti-EE mAb to verify equal recovery of kinases and substrate, shown in the *bottom panel*. Wild type MEK was active when expressed in the presence of *raf*, but not in the presence of the inactive mutant *raf*. Mutation of both sites to alanine codons completely prevented both basal MEK activity and *raf* activation. Mutation of individual serine codons to alanine resulted in partial activation. Autophosphorylation of MEK parallels phosphorylation of the MAPK substrate.

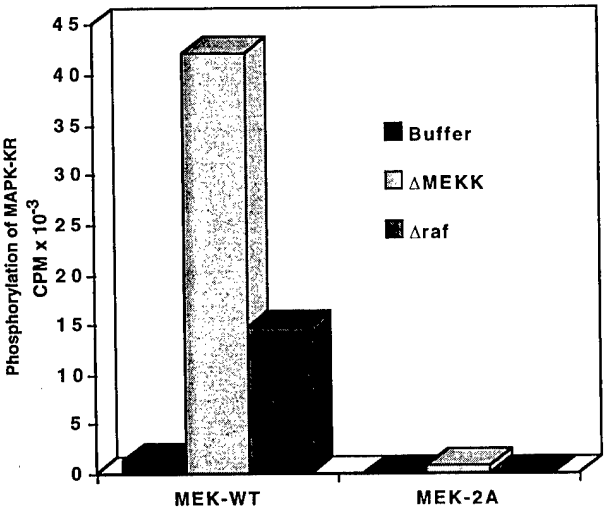

FIG. 5. **Ser^{218} and Ser^{222} are required for activation of MEK *in vitro* by MEKK or *raf*.** Immunopurified MEK-WT or MEK S218A/S222A mutant was activated *in vitro* with purified ΔMEKK, Δ*raf*, as described under "Experimental Procedures" using MAPK-KR as substrate. After SDS-PAGE and autoradiography, labeled MAPK was excised from the gel and quantified by Cérenkov counting. MEK-WT, but not the S218A/S222A, mutant was efficiently activated by ΔMEKK and Δ*raf*.

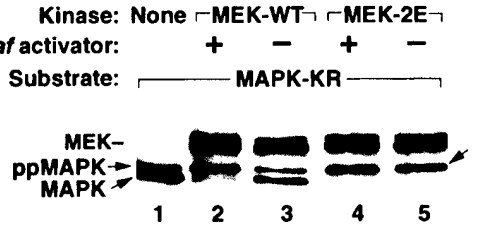

FIG. 6. **MEK-2E allele is constitutively active.** EE epitope-tagged MEK-WT or MEK-2E proteins were expressed together in cells with untagged Δ*raf* or the kinase-inactive Δ*raf*-KR mutant (designated + or − *raf* activator). Activity of the immunopurified MEK was detected using inactive MAPK-KR as substrate, detecting phosphorylation of MAPK by upward mobility shift on SDS-PAGE. MAPK is mostly unphosphorylated when incubated without MEK protein (*lane 1*). Incubation of the MAPK-KR substrate with Δ*raf*-activated MEK-1 results in nearly complete conversion of MAPK to the phosphorylated form (*lane 2*), but little conversion if MEK-1 is co-expressed with the inactive *raf* allele (*lane 3*). The MEK-2E mutant protein is also able to effect complete conversion of the MAPK-KR substrate to the phosphorylated form, either when expressed with (*lane 4*) or without (*lane 5*) active *raf* kinase. Thus MEK-2E activity is independent of upstream activation.

to be active independent of activation by phosphorylation by upstream kinases. To test this hypothesis, we constructed a mutant allele of MEK that contains the two mutations S218E and S222E, termed MEK-2E. Immunopurified MEK-2E was found to be catalytically active (see Fig. 6) but a more important question was whether the activity of the MEK-2E protein requires activation by upstream kinases.

In the experiment shown in Fig. 6, we expressed MEK-WT or MEK-2E together in cells with either Δ*raf* or the kinase-inactive Δ*raf*-KR mutant (neither *raf* allele was epitope tagged in this experiment). We then measured the ability of the immunopurified MEK to phosphorylate inactive MAPK-KR, detecting phosphorylation of MAPK by upward mobility shift on SDS-PAGE. Approximately 90% of immunopurified MAPK substrate is unphosphorylated (*lane 1*) when incubated without MEK protein. Incubation of the MAPK-KR substrate with Δ*raf*-activated MEK-1 results in nearly complete conversion of MAPK to the phosphorylated form (*lane 2*), but little conversion of MAPK is seen if MEK-1 is co-expressed with the inactive *raf* allele, demonstrating that activity of wild type MEK-1 protein is dependent upon activation by the co-expressed *raf* kinase. The MEK-2E mutant protein is also able to effect complete conversion of the MAPK-KR substrate to the phosphorylated form, but in contrast to the wild type MEK protein, MEK-2E is active when expressed either with or without active *raf* kinase

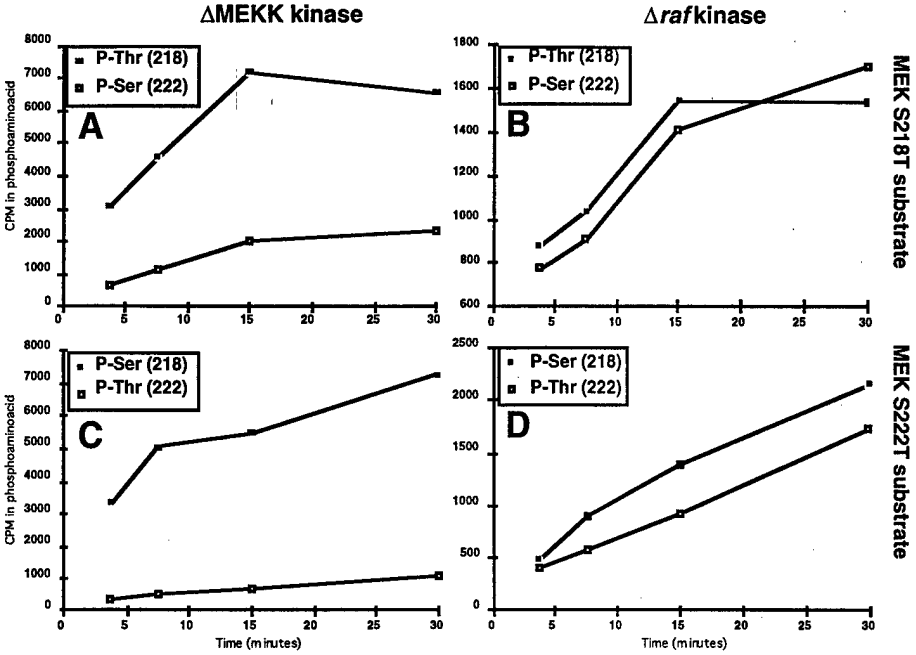

FIG. 7. **Time course of MEKK and *raf* phosphorylation of each activating residue.** To measure phosphorylation of MEK on codons 218 and 222, MEK S218T or S222T mutants were constructed, together with the inactivating K97R mutation of MEK-1. Each mutant MEK-1 protein was expressed separately and then immunopurified and reacted together with ΔMEKK or Δraf kinases. Parallel reactions were stopped after increasing time with SDS sample buffer and analyzed by SDS-PAGE. Radiolabeled MEK protein was subjected to phosphoamino acid analysis, and the amount of radiolabel associated with phosphoserine or phosphothreonine was quantified using an AMBIS β-detector. Four experiments are shown using either MEK S218T substrate (*A* and *B*) or MEK S222T (*C* and *D*) reacted with either ΔMEKK (*A* and *C*) or Δ*raf* (*B* and *D*) for the time shown. The amount of radiophosphoamino acid detected arising from each codon (either phosphoserine or phosphothreonine) is plotted separately. MEKK phosphorylates the residue at codon 218 more rapidly than the residue at codon 222 regardless of the hydroxy amino acid at that codon position. In contrast, Δ*raf* kinase phosphorylates both codons approximately equally at all time points.

(*lane 5*). Thus MEK-2E activity is independent of upstream activation.

MEKK and raf Have Distinct Specificity for Site Phosphorylation—The experiment shown in Fig. 3 suggests that phosphorylation of MEK-1 by *raf* results in approximately equal phosphorylation on serines at codons 218 and 222, whereas MEKK phosphorylates codon 218 preferentially. However, this distinction could reflect differences in the completion of the phosphorylation reaction rather than differences in the actual site preferences. Therefore we sought means of observing the kinetics of phosphorylation of each codon separately.

We first considered using synthetic peptide substrates to measure the site specificity of MEKK or *raf*. However, versions of MEK-1 containing large (50–100 codons) deletions distant to the activating sites have been completely unphosphorylated by MEKK or *raf*.[2] We interpret this to mean that an intact MEK protein is required for recognition of MEK-1 as a substrate by these kinases. It thus seems unlikely that peptide substrates could give meaningful results.

To measure phosphorylation of MEK on each of the two codons, we instead utilized MEK S218T and S222T mutations, together with the inactivating KR mutation. The reciprocal threonine mutations are important for measuring potential differences in *codon* specificity *versus* preference for serine over threonine residues. MEK S218T or S222T mutants were expressed separately and then immunopurified and reacted together with ΔMEKK or Δ*raf* kinases for increasing time periods. Radiolabeled MEK protein was subjected to phosphoamino acid analysis, and the amount of radiolabel associated with phosphoserine or phosphothreonine was quantified using an AMBIS β-detector.

Fig. 7 shows the analysis of the four experiments using the two substrates and the two kinases. MEKK was found to phosphorylate the residue at codon 218 more rapidly than the residue at codon 222 in reactions with both mutant MEK proteins (*A* and *C*). At later time points, phosphorylation on codon 218 plateaus and phosphorylation at codon 222 increases slightly. In contrast, Δ*raf* kinase (*B* and *D*) phosphorylated both codons approximately equally at all time points. When codon 222 was substitute with threonine, slightly less radioactive phosphothreonine was detected with either kinase. This may reflect that threonine is a slightly less preferred residue for both kinases. It is clear, however, that phosphorylation of MEK by MEKK and *raf* is biochemically distinguishable, although we have not detected biological consequences of this differentially phosphorylated MEK proteins.

DISCUSSION

Identification of the sites of activation of MEK-1 clarifies the role of MEKK and *raf* in transduction of cellular growth signals. The two sites of activating phosphorylation lie within a domain of MEK-1 between kinase domains VII and VIII (18). This activation domain of MEK-1 is conserved between MEK variants and species homologs. Fig. 8*A* shows the alignment of the comparable region of many MEK homologs across diverse species. In all cases, the 2 serines identified as MEK-1 activation sites are preserved, although in yeast and *Xenopus* the second serine is changed to a threonine residue.

Fig. 8*B* depicts the analogous regions of several serine-threonine protein kinases for which the sites of activating phosphorylation are known. In all examples found, activating phosphorylation also occurs between conserved kinase domains VII and VII. For the analogous activating region of MAPK, this region lies in a solvent-exposed portion of the protein, which has been termed the "activating lip" (34) and which may partially obstruct a substrate binding pocket. Spacial conservation of the

[2] M. Yan, unpublished observations.

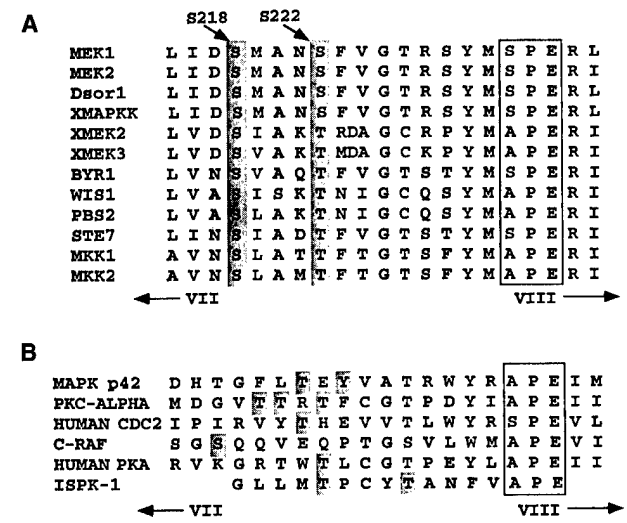

FIG. 8. **Comparison of the activation domain in homologs of MEK-1 and in other serine/threonine kinases.** *A*, amino acid sequences of MEK-1 and homologous proteins are compared in the region of the activating phosphorylation sites of MEK-1. *Shaded boxes* indicate serine or threonine residues conserved at the sites of MEK activation. Amino acid sequences of rat MEK-2 (MAP kinase kinase-2) (4) *Drosophila* Dsor-1 (19), *Xenopus* MAPKK (20), *Xenopus* MEK-2 and MEK-3 (21), *Schizosaccharomyces* byr-1 (22) and wis-1 (23), *Saccharomyces* PBS-2 (24), STE7 (25), and MKK1 and MKK2 (26) were from published sources. *B*, regions of several serine/threonine protein kinases between conserved domains VII and VIII are compared in which the kinases are known to be stimulated by phosphorylation in this region. *Shaded boxes* show sites of phosphorylation. Published sources identifying activating sites were used for MAPK (27), PKC-α (28), cdc2 (29, 30), c-*raf* (31), PKA (32), and ISPK-1 (33).

sites of activating phosphorylation suggests that this mode of regulation of kinase activity is strongly conserved, especially among kinases within signal transduction cascades. Activation sites in other kinases might thus be inferred by homology to this region.

The constitutive activity of our MEK-2E allele strongly supports the identification of codons 218 and 222 as sites of activating phosphorylation on MEK. Furthermore, this allele could prove to be a valuable reagent for analysis of signal transduction events, since it almost certainly contributes a continuous MEK-1 signal to the cell. We have recently constructed cell lines that express the MEK-2E allele in a regulated fashion and are beginning to measure the effects of this allele on growth signaling in cells. It should be noted that our strategy of substituting acidic residues in place of activating phosphorylation sites is not always successful. As a pertinent example, we have detected no activity in alleles of MAPK (obtained from Michael Weber) containing substitutions of glutamic acid for either the phosphorylated threonine, or tyrosine residues, or both.

Although MEK-1 is clearly activated by *raf* and MEKK, it is also capable of phosphorylating itself, as documented by several groups (20, 35). Using our S218T and S222T mutants we have confirmed that MEK-1 autophosphorylates both codons 218 and 222 and also phosphorylates uncharacterized threonine and tyrosine residues.

Our studies have shown clearly that phosphorylation of MEK-1 by MEKK and *raf* are not identical, with MEKK strongly preferring codon Ser^{218} as a site of phosphorylation. However, we do not know if this difference is reflected in biological differences in the signaling process effected by these two kinases. Using phosphorylation and activation of MAPK as a measure of MEK-1 function, both MEKK and *raf* seem equally able to activate MEK-1. The differences we observe in site preference might, however, reflect biological differences in signaling in several ways. For example, the two distinctly phosphorylated forms of MEK could recognize different substrates other than MAPK. Under this scenario doubly phosphorylated MEK (activated by *raf*) could phosphorylate an unknown substrate *critical for cell transformation* that is not recognized by MEKK activated MEK-1. Alternatively, one of the differently phosphorylated MEK-1 forms could remain activated longer within the cell. Since phosphatase(s) that inactivate MEK-1 have not been characterized, it is possible that a separate phosphatase is responsible for dephosphorylating each residues. If this were true, termination of the two activating events might be separately regulated. Since our data indicate that singly phosphorylated MEK-1 remains partially activated (see Fig. 4), a phosphatase specific for codon 218 could specifically negate signals arising from MEKK activation while leaving the signal arising from *raf* partially intact.

Irrespective of mechanism, the differences between MEK-1 signal transduction effected by MEKK or *raf* is significant for one overriding reason: *raf* has clearly been identified as a component of an oncogenic kinase cascade, whereas MEKK has not. Alternative phosphorylation of MEK-1 protein, resulting in similar yet distinct activation of MEK-1, could be a means by which oncogenic *versus* non-oncogenic growth signals are propagated.

Since this work was completed, a report identifying MEK codons 218 and 222 as substrates for *raf* has been published (36).

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Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1

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A KINASE distinct from the MEK activator Raf[1–3], termed MEK kinase-1 (MEKK), was originally identified by virtue of its homology to kinases involved in yeast mating signal cascades[4]. Like Raf, MEKK is capable of activating MEK *in vitro*[4,5]. High-level expression of MEKK in COS-7 cells[4] or using vaccinia virus vectors[5] also activates MEK and MAPK, indicating that MEKK and Raf provide alternative means of activating the MAPK signalling pathway. We have derived NIH3T3 cell sublines that can be induced to express active MEKK. Here we show that induction of MEKK does not result in the activation of MAPK, but instead stimulates the stress-activated protein kinases (SAPKs)[6–8] which are identical to a Jun amino-terminal kinase[9,10]. We find that MEKK regulates a new signalling cascade by phosphorylating an SAPK activator, SEK1 which in turn phosphorylates and activates SAPK.

Stably transfected NIH3T3 subclones express MEKK in response to isopropyl-β-D-thiogalactosidase (IPTG) (Fig. 1*a*) but MAPK activity remains unchanged (Fig. 1*b*). In contrast, SAPK activity is increased six- to eightfold in MEKK-inducible cell lines but not in the parent NIH3T3 cells. These MEKK-expressing cells are able to activate MAPK in response to some mitogenic signals, because treatment with phorbol ester increases MAPK activity in each of these clones (as well as in NIH3T3 cells), whereas SAPK activity is unaffected (Fig. 1*c*). Increased SAPK activity is evident by 3 hours and maximal after 12 hours of induction; MAPK activity is unchanged throughout the 23-hour incubation with inducer (Fig. 1*d*). Together these results indicate that, in contrast to the presumed role of MEKK in activating MEK and MAPK, MEKK acts instead to activate SAPKs. Expression of truncated ΔMEKK in these clones resulted in six- to eightfold inhibition of growth rate compared with parental NIH3T3 cells.

We modelled activation of SAPK by MEKK using cloned genes and purified proteins expressed using vaccinia virus vectors[11,12]. MEKK induced electrophoretic retardation of SAPK, which was suggestive of quantitative phosphorylation (Fig. 2*a*), and also increased the amount of phosphotyrosine in SAPK and activated its Jun N-terminal kinase activity. Thus, in this overexpression model as well as in the inducible cell line, MEKK expression results in activation of the SAPK pathway.

We considered the possibility that activation of SAPK occurred as a consequence of activation of the MEK and MAPK cascade. To stimulate MAPK independently of MEKK, we used activated Raf and a constitutively active allele of MEK1 termed MEK 2E (ref. 5). Both Raf and MEK 2E were able to induce phosphorylation of coexpressed MAPK (Fig. 2*b*). Neither of these MAPK activators induced phosphorylation of SAPK, indicating that the SAPK activation pathway is effectively insulated from the MAPK pathway.

MEKK was unable to phosphorylate SAPK *in vitro* (below). We therefore tested whether MEKK activated the newly identified SAPK activator, SEK1 (ref. 13), whose sequence is similar to MEK1. Immunopurified MEKK (but not the inactive mutant

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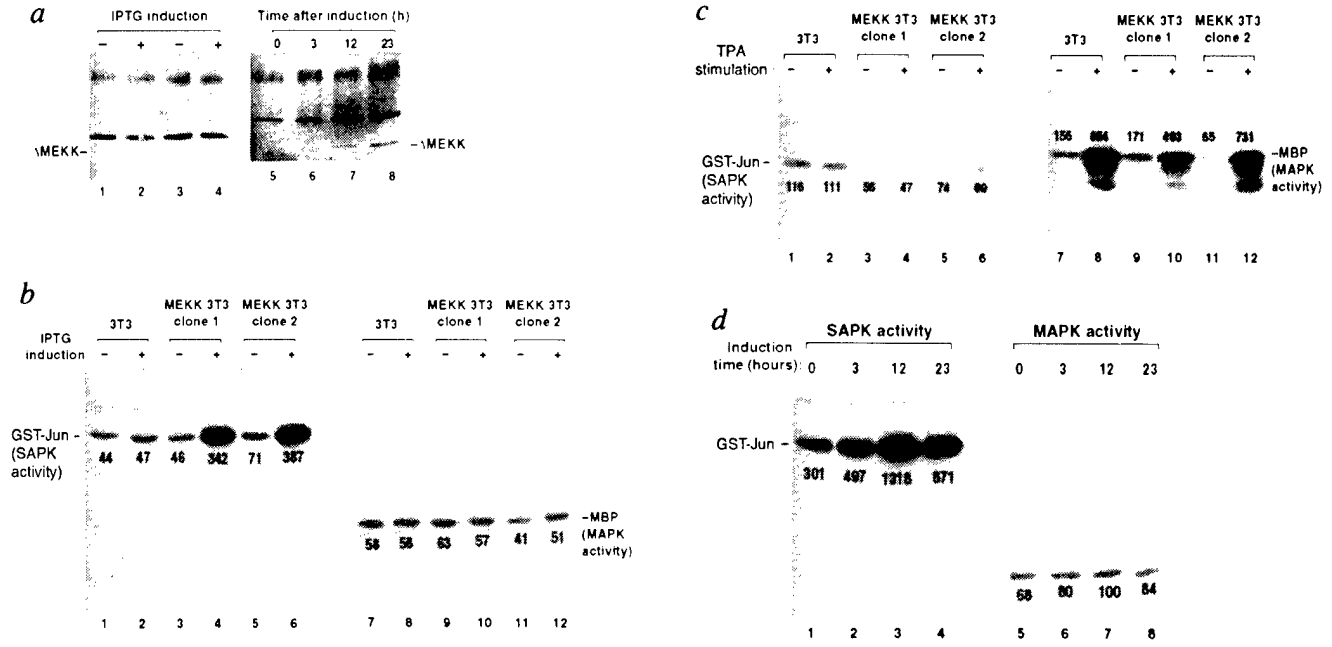

FIG. 1 *a*, MEKK expression in NIH3T3 cells. Epitope-tagged truncated MEKK (ΔMEKK) was detected in MEKK clone 1 cells (lanes 1 and 2) or in MEKK clone 2 cells (lanes 5–8) but not in a control clone (lanes 3 and 4) treated with IPTG for 24 h (lanes 2 and 4) or at the indicated times (lanes 6–8). *b*, MAPK and SAPK activity in MEKK-inducible cell lines. Numbers below labelled bands indicate c.p.m. of radioactivity in substrates. SAPK activity, but not MAPK activity, was increased in response to MEKK expression. *c*, Functional MAPK signalling in NIH3T3 cells and MEKK-expressing subclones after stimulation (+) with 250 ng ml^{-1} TPA. *d*, Time course of induction of SAPK activity in MEKK 3T3 clone 2 cells after IPTG treatment. MAPK activity throughout this period remained unchanged, whereas SAPK activity was increased even at the 3-h time point, when MEKK expression could not yet be detected. MEKK expression in cells used here is shown in *a*, lanes 5–8.

METHODS. The EE epitope-tagged[5] C-terminal 320 amino acids of MEKK1 (ΔMEKK) was expressed in NIH3T3 cells using the lacSwitch promoter (Stratagene). ΔMEKK was induced in cell clones with 1 mM IPTG and detected by immunoprecipitation and immunoblotting using the anti-EE monoclonal antibody (mAb). MAPK and SAPK activity was determined using polyclonal antibodies recognizing a C-terminal peptide of p42MAPK or a p54SAPK–GST fusion protein. Immune complexes containing protein from 10^5 cells were reacted with 0.5 μg GST–Jun (amino acids 5–89; ref. 8) for SAPK, or MBP (Sigma) for MAPK in 20-μl reactions (50 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 15 μM ATP, 5 μCi [^{32}P-γ]ATP), for 30 min at room temperature. Radioactivity was quantified using an AMBIS β-detector.

MEKK(K → R) rapidly phosphorylated a glutathione-*S*-transferase(GST)–SEK fusion protein on serine and threonine residues (Fig. 3*a*) but failed to phosphorylate a SEK mutant in which the two residues equivalent to the sites of activation in MEK were mutated. Phosphorylation of GST–SEK activated the SAPK activity of GST–SEK. Thus, SEK is a substrate of MEKK and phosphorylation by MEKK is sufficient to activate SEK. MEKK expression also activates SEK *in vivo* (Fig. 3*b*). Activation of SAPK by MEKK requires functional SEK because coexpression of a dominant inhibitory allele of SEK blocks activation of SAPK by MEKK (Fig. 3*c*).

Coexpression of full-length MEKK protein is able to effect phosphorylation of SEK and activation of SAPK (Fig. 4*a*), similar to the activation induced by truncated MEKK. The high activity of full-length MEKK protein during overexpression suggests that a cellular activity might regulate natural MEKK expressed at lower levels. This result is in contrast to Raf, which displays low levels of kinase activity unless truncated[14].

Our results demonstrate complete reconstitution of a kinase cascade, beginning with MEKK, that phosphorylates and activates SEK, which subsequently phosphorylates and activates SAPK. Each component of this cascade is functionally parallel

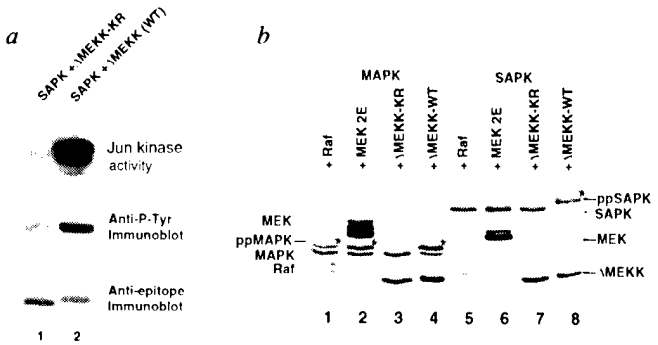

FIG. 2 Activation of SEK–SAPK pathway by coexpression of ΔMEKK using vaccinia virus vectors. *a*, Epitope-tagged SAPK was expressed with either the untagged inactive K → R mutant of ΔMEKK (lane 1) or wild-type ΔMEKK (lane 2). Coexpression of active MEKK resulted in mobility shift of SAPK detected by anti-epitope immunoblotting (bottom panel) and also increased tyrosine phosphorylation of SAPK detected in anti-epitope immunoprecipitates (middle panel). SAPK activity was also strongly elevated, reflected by phosphorylation of GST–Jun(5–89) using anti-epitope immunoprecipitates (top panel). *b*, epitope-tagged MAPK (lanes 1–4) or SAPK (lanes 5–8) was expressed with epitope-tagged forms of truncated active Raf (lanes 1, 5), constitutively active MEK 2E (lanes 2, 6), ΔMEKK(K → R) mutant (lanes 3, 7) or ΔMEKK wild type (lanes 4, 8), and detected in whole cell lysates using anti-epitope western blot. Activation of both MAPK and SAPK is identifiable by the appearance of bands with delayed mobility, indicated by stars. pp prefix, phosphorylated protein forms. Raf and active MEK 2E are able to activate MAPK, but not SAPK, thus the SAPK pathway is insulated from the MAPK pathway. MEKK is able to activate MAPK in this overexpression system, though it is not when expressed at lower levels (Fig. 1). Of the kinases tested, only MEKK is able to activate SAPK.

METHODS. The N-terminal EE-epitope-tagged p54SAPKα1, and untagged ΔMEKK were expressed using the vaccinia virus expression system and the plasmid pTM1 (ref. 12). Phosphotyrosine was detected using mAb 4G10 (UBI) to probe anti-EE immunoprecipitates. Jun kinase was assayed as for Fig. 1. Kinase expression and blotting has been described[5].

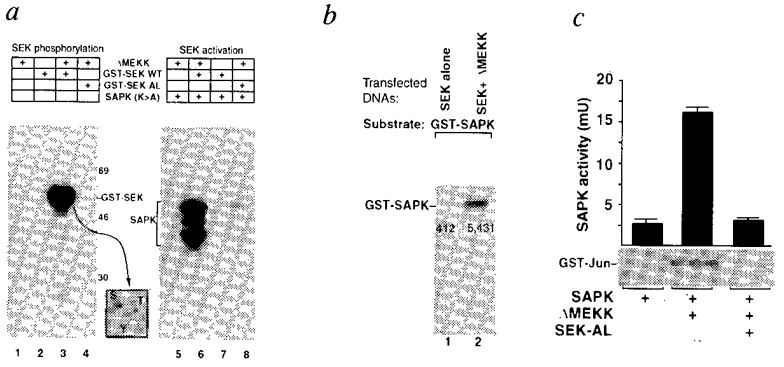

FIG. 3 *a*, MEKK phosphorylates and activates of SEK *in vitro*. Immunopurified ΔMEKK phosphorylated wild-type GST–SEK (lane 3) on serine and threonine (see phosphoaminoacid analysis, inset), but not mutant GST–SEK protein lacking the two phosphorylation sites (lane 4). GST–SEK1 phosphorylated by MEKK *in vitro* acquired SAPK kinase activity, as shown after secondary reaction with inactive (K>A) mutant thrombin-cleaved GST–SAPK with radioactive ATP (lane 6). Mutant GST–SEK protein lacking phosphorylation sites (lane 8) or reactions without either MEKK or SEK did not allow phosphorylation of SAPK. *b*, MEKK activates SEK1 *in vivo*. Epitope-tagged SEK expressed in CV1 cells using a CMV expression vector (lane 1) became activated by coexpression of ΔMEKK lacking the epitope tag (lane 2). Anti-epitope immunocomplexes were assayed for SEK activity using GST–SAPK as substrate (see Fig. 1 legend). *c*, SAPK activation by MEKK requires SEK1. Epitope-tagged SAPK expressed using SV40-based vectors was activated by coexpression with ΔMEKK. This activation was reversed by triple coexpression of a dominant inhibitory mutant of SEK1 containing (S220A, T224L; SEK AL). METHODS. Epitope-tagged ΔMEKK was expressed, immunopurified and eluted using excess EE peptide[5]. Bacterial SEK–GST fusion protein was purified and reacted *in situ* on glutathione–agarose beads. Activated MEKK was separated from GST–SEK by washing the glutathione beads, and subsequently incubated with SAPK substrate (cleaved from GST by thrombin, and containing an inactivating K55A mutation) in kinase reactions containing [^{32}P]ATP (20 mM ATP total concentration). Inhibition of HA-epitope-tagged SAPK by SEK-S220A, T224L (SEK-AL) was tested in L929 cells using the SV40-based pMT2 vector; 7 μg SAPK, 7 μg ΔMEKK and 15 μg SEK-AL expression plasmids were transfected together with empty pMT2 vector DNA to equalize plasmid mass. SAPK assays represent triplicate measurements using GST–Jun substrate as described[13].

to a component of the MAPK activation pathway: SAPK is analogous to MAPK, SEK analogous to MEK, and MEKK analogous to Raf (Fig. 4*b*). Other evidence suggests that SAPK signalling in response to ultraviolet irradiation[10,15] and tumour-necrosis factor-α[16] lies downstream of Ras. Additionally, dominant inhibitory Ras reduces the activity of MEKK[17] and the MEKK homologue Byr2 associates with Ras1 in yeast[18]. Thus, in parallel to Raf–MEK–MAPK, the MEKK–SEK–SAPK pathway most probably lies downstream of Ras. Cofactors for Ras must exist that contribute specifically to either the mitogenic or stress-response pathways.

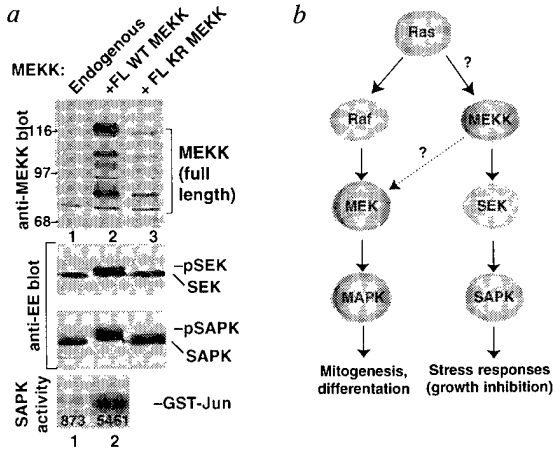

FIG. 4 *a*, Activation of SEK and SAPK *in vivo* by full-length MEKK. Using the vaccinia virus expression system in CV1 cells, SEK1 or SAPK were separately expressed alone (lane 1) or with vectors encoding full-length wild-type MEKK1 (lane 2) or a kinase-inactive (K447R) mutant allele of MEKK1 (lane 3). Expression of several bands related to full-length MEKK (top panel) was detected using chicken polyclonal antibodies raised against bacterially expressed ΔMEKK. Endogenous proteins of 70K, 90K and 110K were also detected (lane 1). Epitope-tagged SEK1 and SAPK1 (middle panels) were detected by immunoblotting. Electrophoretically retarded bands arising from phosphorylation of both SEK and SAPK were observed from coexpression with full-length MEKK, indicating that MEKK thus expressed is constitutively active. SAPK activity was activated by full-length MEKK (bottom panel). *b*, Diagram of separate pathways emanating from Raf and MEKK. Both Raf and MEKK appear to be dependent upon the function of Ras, as described in the text. The Raf–MEK–MAPK pathway is functionally analogous to the MEKK–SEK–SAPK pathway, although the result of stimulation of each pathway is distinct. A dotted arrow from MEKK to MEK reflects the ability of MEKK to phosphorylate MEK *in vitro* and during high-level cell expression. In stable inducible cell lines, MAPK activation by MEKK is not seen, drawing the physiological significance of this path into question. The opposing nature of two signalling pathways both emanating from Ras suggests an important role for factors cooperating with Ras to provide specificity for stimulation of one path versus the other.

Is MEKK able to activate MEK, as originally proposed? When tested *in vitro* or during overexpression[4,5], MEKK is able to phosphorylate and activate MEK. But with the stable MEKK-inducible NIH3T3 cells studied here, MEKK, through SEK, activates SAPK and not MAPK, even though these cells express an intact MAPK activation pathway. MEKK might stimulate MAPK in other cell types, or transiently, although we detected no MAPK activity as early as 3 hours, at which time SAPK activity was raised and MEKK was undetectable. Additionally, activated *Drosophila* MEK (Dsor1) rescues D-Raf null mutants in both the Torso and R7 photoreceptor pathways[19], implicating Raf as the major physiological MEK activator.

Perhaps it is appropriate to consider MEKK versus RAF signalling as analogue rather than binary cell regulation. Depending on the interplay between kinase activities, substrate availability and the intracellular milieu, MEKK might activate SAPK in our experimental systems; other cellular conditions might translate MEKK activity into a variety of mixed signals involving other homologues of MAPK[20], including SAPK. □

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